STUDIES ON PREPARATIVE VERTICAL ZONE ELECTROPHORESIS

With Special Reference to the Isolation of Some Protein Fractions from Individual Human Sera at pH 4–6

by

A. HELGE F. LAURELL

Institute of Biochemistry
Uppsala

UPPSALA 1958

ALMQVIST & WIKSELLS BOKTRYCKERI AB

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PREFACE

My interest in substances containing hexosamine and their distribution in nature was aroused during the tenure of a British Council Scholarship in Professor W. T. J. Morgan's laboratory at the Lister Institute in London 1944–45.

During subsequent years as physician in Professor Nanna Svartz' clinic and laboratory at the Caroline Hospital in Stockholm it was found that the hexosamine serum levels reflected disease activity. Preliminary experiments soon showed that several globulin fractions, if not all, contained hexosamine. In order to analyze the observed changes in hexosamine levels it was necessary to characterize the hexosamine content of different globulin fractions. Such studies started in 1950 in the Institute of Biochemistry, Uppsala, where the development of methods for zone electrophoresis and for chromatography has furnished tools to make possible the analysis of well-defined fractions from individual sera.

The methodological part of this study has benefited greatly from the collected experiences of different aspects of electrophoretic fractionation which have accumulated in the Institute of Biochemistry.

I would like to thank Professor Arne Tiselius for his kind interest and helpful suggestions made during the course of this work, as well as for the excellent laboratory facilities which he placed at my disposal.

I wish to express my sincere gratitude and indebtedness to my teachers, Professor Gunnar Blix, Professor Anders Grönwall, Professor Walter Morgan, Professor Nanna Svartz, and Professor Arne Tiselius.

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INTRODUCTION

At the end of the 19th century experimental evidence had accumulated suggesting that the serum proteins contained bound carbohydrates. Mörner (1893) showed that phenylhydrazine derivatives of carbohydrates could be isolated from serum globulin after acid hydrolysis. The blood "albumose" fraction in serum, which was soluble in distilled water, was known to contain glucosamine, and Bywaters (1909) showed that it was probably identical with seromucoid. Pavy (1907) suggested that the amount of free carbohydrate in serum was probably less than the protein-bound carbohydrate. Wolff (1921) found "albumosemia" in a case of rheumatoid arthritis but also in many other diseases. Grevenstuk (1929) reviewed the entire field of free and protein-bound carbohydrates and by that time had already assembled 546 references. Lustig and Langer (1931), Hewitt (1937), and Rimington (1940) pursued studies on serum glycoproteins during the following years.

This field has been the subject of a renewed interest during the last five years as new methods for isolation and characterization of proteins and glycoproteins have appeared. Naturally these studies have been stimulated by recent observations indicating that marked changes in the distribution of these substances in serum occur in different diseases, and that the levels of the glycoproteins are markedly influenced by some hormones:

Jayle, Said, Gillard, Abdellatif, and Schwob (1946),

Jonsson, Berglund, Hakanson, Hävermark, and Laurell (1950), Kelley, Good, and McQuarrie (1950), Stary, Bursa, Kaleoglu, and Bilen (1950), Videbæck *et al.* (1950),

Wunderly, Karrer, Hugentobler, and Wuhrmann (1951),

Dische and Osnos (1952), Köiw and Grönwall (1952), Laurell (1952), Werner and Odin (1952),

Coburn and Haninger (1954), Greenspan (1954), Jacqueline, Groulade, and Drufovka (1954), Jayle and Boussier (1954), Payne *et al.* (1954), Raynaud, d'Eshougues, Pasquet, and Pasquet (1954), Uzman and Rosen (1954), Wunderly and Piller (1954),

Badin, Schubert, and Vouras (1955), Boas, Bollet, and Bunim (1955), Gamp (1955), Infante Miranda (1955), Kuhns and Crittenden (1955), Mandel, Gorsuch, and Cooper (1955), Turumi, Saito, and Takahashi (1955), Yamakawa and Suzuki (1955),

Bergstermann (1956), Bernasconi (1956, 1957), Gottschalk (1956), Gukelberger and Sonntag (1956), Heremans (1956), Kalliomäki (1956), Keiding (1956), Kerby (1956), Olhagen (1956), Rosenlund (1956), Schmid (1956), von Studnitz (1956), von Studnitz and Berezin (1956),

Almquist and Lausing (1957), Bollet, Rozlim and Simpson (1957), Böhm, Ross, and Baumeister (1957), Kuhn (1957), Lagrue, Rahman, and Mozziconacci (1957), Larsen and Bent-Hansen (1957), Laurell, Laurell, and Waldenström (1957), Laurell and Nyman (1957), MacLagan (1957), Müller-Eberhard, Kunkel, and Franklin (1957), Niaussat-Ansault (1957), Svennerholm (1957),

Bodman (1958), Deiss (1958), Ledvina (1958), Moschides, Stefanini, Magalini, and Kistner (1958), Nilsson and Yamashina (1958), and Schultze (1958*a*).

Protein-bound carbohydrates have recently been reviewed by Goa (1955), Kent and Whitehouse (1955), Winzler (1955), Sonnet (1956), Südhof and Kellner (1957), Blix (1958), Blix and Gardell (1958), Laurent (1958), and Schultze (1958*b*).

West, Clarke, and Kennedy (1938), Winzler, Devor, Mehl, and Smyth (1948) observed increased serum level of protein-bound hexosamine in some cases of rheumatoid arthritis.

The hexosamine content of electrophoretically separated serum proteins was first studied by Blix, Tiselius and Svensson (1941). They found that the alpha and beta globulins contain more hexosamine than do gamma globulin or albumin. In a case of pneumonia, however, the carbohydrate content of the alpha and beta globulins and also that of the albumin fraction was markedly increased.

During administration of ACTH to patients with rheumatoid arthritis Jonsson, Berglund, Hakanson, Hävermark and Laurell (1950) found that initially elevated hexosamine values showed a decrease concomitant with a temporary, artificial remission. The same type of change was observed after administration of cortisone (Laurell, 1952). These preliminary results have been extended and confirmed independently by Jiménez-Diaz, Aguirre, and Arjona (1953) who found that the hexosamine level was a good indicator of disease activity in rheumatoid arthritis, and who also observed a fall in these elevated levels to normal values during the administration of ACTH or cortisone. Shetlar *et al.* (1953), Bonomo (1953), Bonomo, Sala, Amira and Ballabio (1956) also found serum polysaccharide levels useful for the evaluation of the degree of activity of rheumatic diseases. They also confirmed that the serum-bound carbohydrates decreased during the administration of cortisone.

Early studies of blood from cases with severe rheumatoid arthritis had shown that it contained a conspicuous amount of "fibrin", an observation which was known already to Berzelius (1849).

Electrophoretic studies of serum proteins from patients with severe active rheumatoid arthritis, Dole and Rothbard (1947), Svartz and Olhagen (1948), had often shown increased alpha-2 globulin values and occasionally an increase in alpha-1, beta and gamma globulins. Increased alpha globulin values are, however, not only found in severe rheumatoid arthritis but also in a number of unrelated diseases such as advanced tuberculosis, cancer, and chronic infections, Wunderly, Karrer, Hugentobler, and Wuhrmann (1951), Jayle (1956).

Petermann and Hogness (1948) reported electrophoretic studies at pH between 4 and 4.5 of serum proteins from patients with neoplastic disease. Their study indicated that electrophoresis at low pH could be used for differentiation of the rather ill-defined mucoprotein fraction in serum. This fraction is left in solution by perchloric acid and precipitated by phosphotungstic acid. It is largely identical with the fractions studied by Bywaters (1909) and Rimington (1940). The serum mucoproteins have recently been studied extensively by Winzler (1955).

Winzler, Devor, Mehl and Smyth (1948), Hakanson and Luft (1949), Jonsson *et al.* (1950), Kelley, Good, and MacQuarrie (1950) showed that ACTH administered to patients with rheumatoid arthritis decreased the elevated non-specific hyaluronidase inhibitor. Glick, Good, Kelley, Winzler, and Mehl (1949) showed that the unspecific hyaluronidase-inhibitor was not identical with the mucoprotein.

Mehl, Golden, and Winzler (1949) pursued electrophoretic studies of some human sera at pH 4.5. Two fractions were observed with negative charge at pH 4.5 in acetate buffer and they were called M1 and M2. M1 was found to be a homogeneous mucoprotein and it was shown to be a subfraction of the alpha-1 globulin. M2 appeared to be associated with increase in alpha-2 globulin. However, one patient with Kimmelstiel–Wilson disease and increased alpha-2 was found without a notable increase in M2. The studies indicated that electrophoresis at pH 4.5 might serve to differentiate between different forms of alpha-2 elevations.

Laurell (1951) found changes in the electrophoretic serum patterns obtained at pH 4.5 when serum from a patient with rheumatoid arthritis was studied before, during, and after the administration of ACTH (Fig. 1). The peak corresponding to the M1 boundary was resolved into two peaks. This phenomenon was most marked in the M1 descending boundary if the serum concentration was increased (Fig. 1). Changes were also observed in the M2 region of the electrophoretic serum patterns especially in the descending side of the M2 boundary. Electrophoretic patterns obtained from the serum of another patient with severe rheumatoid arthritis and

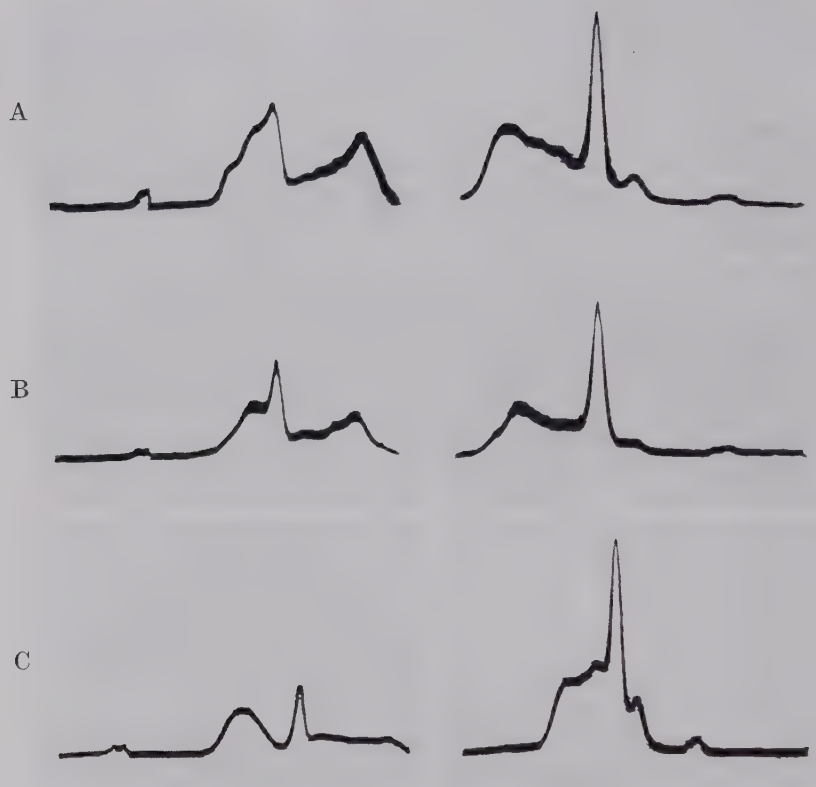


FIG. 1. Electrophoretic boundary patterns obtained with serum from a patient with rheumatoid arthritis.

Sodium acetate buffer pH 4.5, ionic strength $\mu = 0.1$. Descending side for negatively charged components to the left, ascending side for negatively charged components to the right. *A* and *B* were obtained after 200 min. at 20 mA. The protein concentration was 1.5 % (w/v). *A* during an active state of the disease, *B* during a cortisone-induced remission. The protein concentration in *C* is about 2.5 % (w/v). *C*-1 was obtained after 280 min. at 15 mA, *C*-2 after 135 min. at 15 mA. *C*-1 demonstrates clearly the electrophoretic splitting of the M1 fraction.

patterns obtained from a "normal" individual at pH 4.5 are shown in Fig. 2. The electrophoretic diagrams in both figures should be compared with those obtained by Mehl and Golden (1950).

Attempts to determine the hexosamine content in M1 fractions obtained in free electrophoresis were unsuccessful because of the small yield of M1 isolated from individual sera.

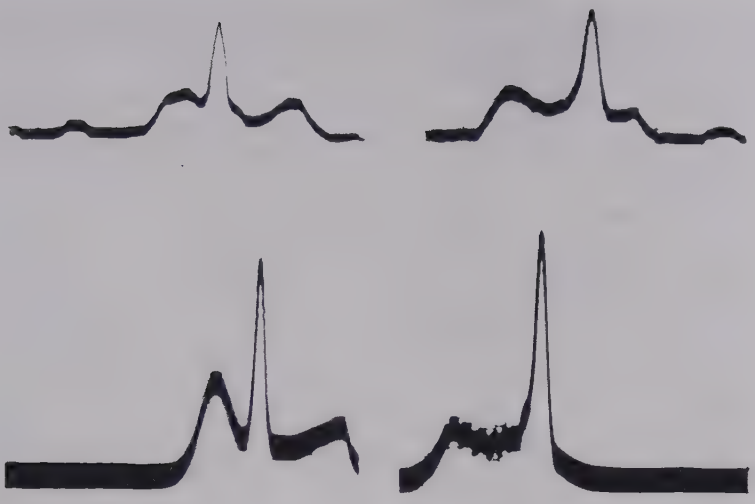


FIG. 2. Electrophoretic boundary patterns obtained with a serum from a patient with severe rheumatoid arthritis (*A*), and with serum from an apparently healthy person (*B*). Acetate buffer pH 4.5, $\mu = 0.1$. Protein concentration 1.5 % (w/v). *A*, 180 min. 17 mA; *B*, 180 min. 20 mA.

The lack of enantiography in the M2 region (Fig. 1) should be observed. The discrepancy between the ascending and descending boundary may partly be due to a reversible equilibrium between albumin and some of the constituents of the solution. Interactions of this type have been studied by Longsworth and MacInnes (1942) and Alberty (1953). As pointed out by Mehl and Golden (1950) a further complication in the interpretation arises from the fact that the δ- and ε-boundaries are to be found in this region between M2 and albumin. Its magnitude is a function of the ratio protein ion to buffer ion. For a complete analysis of the complicated changes taking place in the M2 boundaries all the components present ought to be isolated and studied separately with the buffer used. Cann and Phelps (1957) have recently systematically studied the interaction between serum albumin and undissociated acetic acid in acetate buffers.

About 60 sera from patients with various diseases were investigated by the author using moving boundary electrophoresis at pH 4.5. Severe rheumatoid arthritis never failed to show conspicuous elevation in the M1 and M2 fractions. Such elevations are however found in many other conditions, e.g. amyloidosis.

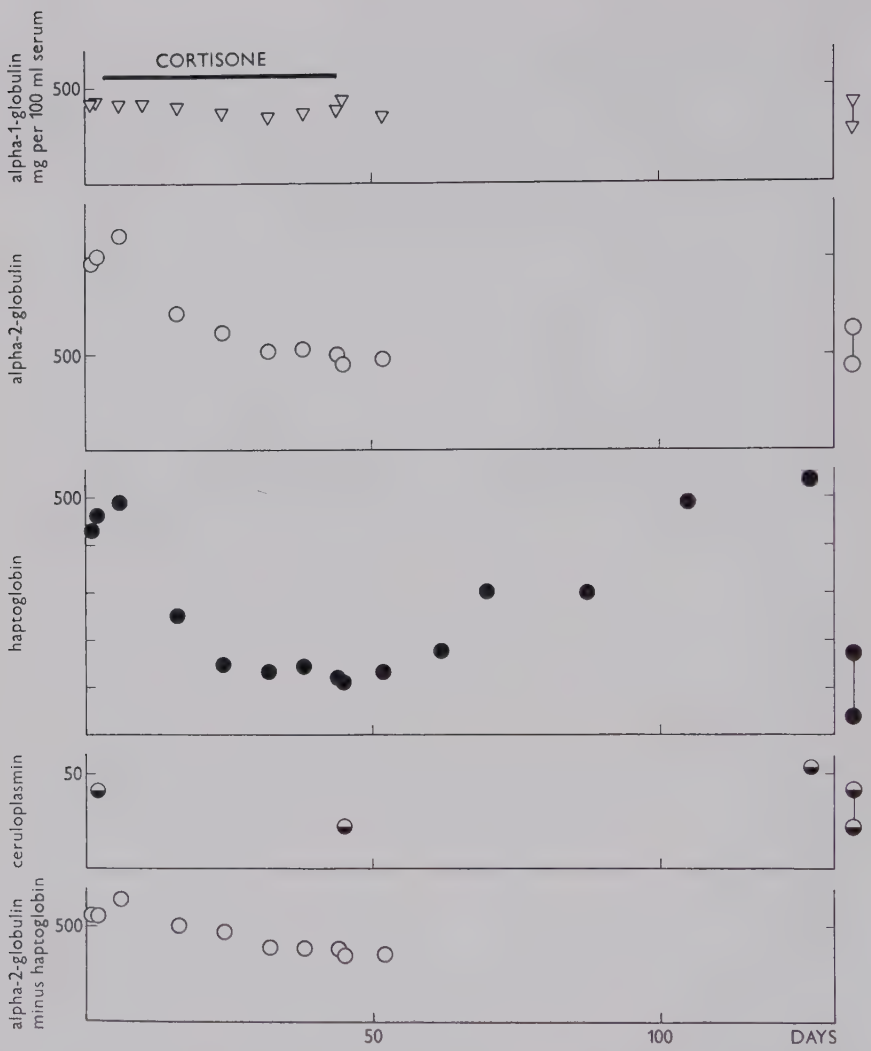


FIG. 3. Values for alpha-1 globulin (▽), alpha-2 globulin (○) and the alpha-2 subcomponents haptoglobin (●) and ceruloplasmin (◒) in a patient with severe rheumatoid arthritis before, during and after cortisone administration. Values for the difference, alpha-2 globulin minus haptoglobin, are at the bottom of the figure. The normal mean value ($\pm 2 \times$ S.D.) found with the same methods is shown to the right of the figure.

The adaptation by Köiw and Grönwall (1952) to filter paper electrophoresis of the McManus technique for staining polysaccharides has greatly facilitated the study of the distribution of protein-bound carbohydrates. This technique is applicable for semiquantitative estimation of protein-

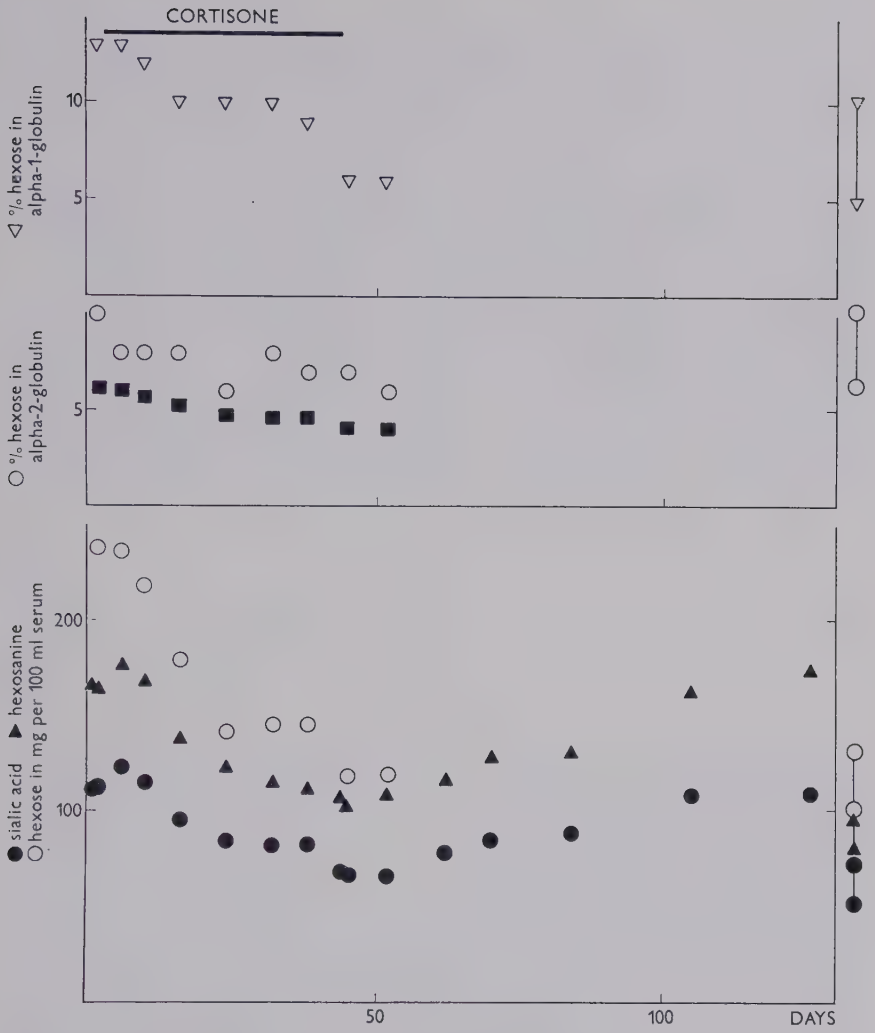


FIG. 4. Values for the ratio carbohydrate to protein in alpha-1 (▽) and alpha-2 globulin (○) in the same patient with rheumatoid arthritis as in Fig. 3. Calculations based on hexose content (Laurell, 1956). Total carbohydrate ratio (■) as obtained from the sum of hexose, hexosamine, and sialic acid values is given in per cent of the total protein. The lower figure gives values for sialic acid (●), hexosamine (▲), and hexose (○) content of serum before, during, and after the administration of cortisone. The normal mean value ($\pm 2 \times$ S.D.) is shown to the right.

bound carbohydrates even though the method lacks chemical specificity for carbohydrates. Good agreement with determinations made by other methods has been obtained by many workers and Björnesjö (1955) found that the intensity of the periodic acid-Schiff (PAS) staining is correlated

to the total amount of protein-bound polysaccharides and that the variations in composition of the polysaccharides are of less importance. C. B. Laurell and Skoog (1956) found a method for eluting the PAS stain from different paper segments.

A group of patients with rheumatoid arthritis was studied with this method by Laurell (1956). Values for the distribution of protein and carbohydrate in different serum fractions before, during, and after the administration of ACTH or cortisone were obtained by this method. Variations observed have significance only if related to the normal mean value as obtained with the same technique. Changes obtained in alpha-1 and alpha-2 globulin levels, in haptoglobin, and ceruloplasmin during the administration of cortisone are shown in Fig. 3. Alpha-2 globulins are initially increased and decrease to the normal range of variation during the administration of the hormone. Pronounced changes were found in the haptoglobin levels. Jayle (1956) observed high haptoglobin levels in rheumatoid arthritis. The difference between alpha-2 globulin and haptoglobin values was essentially independent of time and this demonstrates that the increase in alpha-2 content of serum in this case is largely due to the presence of haptoglobin (Fig. 3). The variation in protein-bound hexose, hexosamine, and sialic acid[1] encountered in the same patient is shown in Fig. 4. A rough measure of the "total protein-bound carbohydrate" in serum is obtained by adding the values obtained for hexose, hexosamine, and sialic acid. The "total carbohydrate" to protein ratio is found in Fig. 4. The ratio alpha-1 carbohydrate to protein decreases during the administration of the cortisone indicating that a carbohydrate rich subcomponent of alpha-1 decreases.

Laurell reported at the Ciba Symposium on Paper Electrophoresis 1955 that the high carbohydrate content of the gamma globulin fraction in rheumatoid sera was unaffected by the administration of cortisone or ACTH.

Franklin, Holman, Müller-Eberhard, and Kunkel (1957), Ziff (1957), and Svartz, Carlsson, Schlossmann and Ehrenberg (1958) have recently found that the gamma globulin in cases of rheumatoid arthritis contains a high molecular weight globulin. It is known that the normal heavy gamma globulin component sedimentation constant $S = 19$ (Müller-Eberhard, 1956; Schultze, 1958*b*)—has a higher content of protein-bound carbohydrate than has the $S = 7$ gamma globulin. These recent findings may explain the high carbohydrate content previously found by Laurell (1956) in the gamma globulin in cases of rheumatoid arthritis (Laurell, 1958).

Markham, Jacobs, and Fletcher (1956) and Bodman (1957) used paper

[1] Studies by Comb and Roseman (1958) indicate that sialic acid from human serum may be enzymatically cleaved to pyruvic acid and N-acetyl-D-mannosamine.

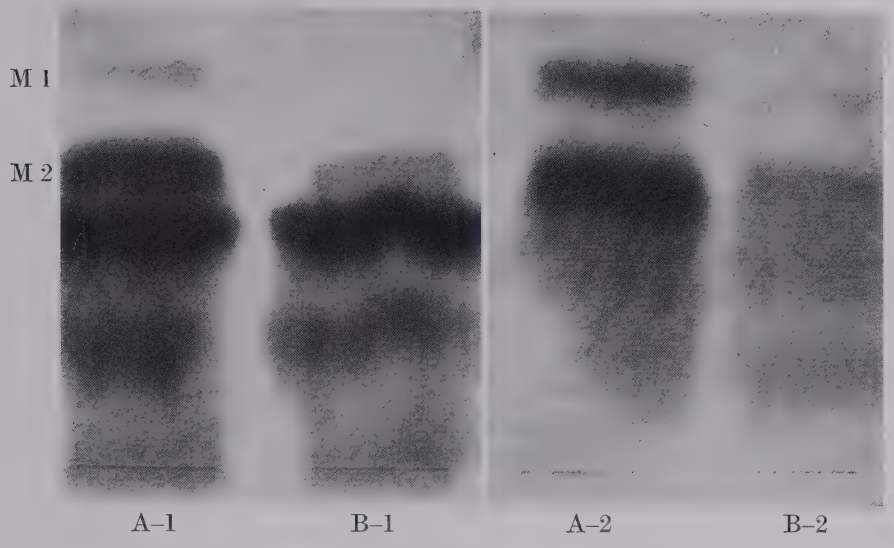


FIG. 5. Paper electrophoresis patterns obtained in citrate-phosphate buffer at pH 4.5 from a patient with rheumatoid arthritis (*A*) compared with patterns from a "healthy" normal person (*B*). *A*-1 and *B*-1 are stained for protein, *A*-2 and *B*-2 for carbohydrate. The carbohydrate rich M 1 and M 2 fractions in front of the albumin in the patient with rheumatoid arthritis (*A*-2) should be compared with the very thin corresponding spots obtained in the "healthy" person (*B*-2).

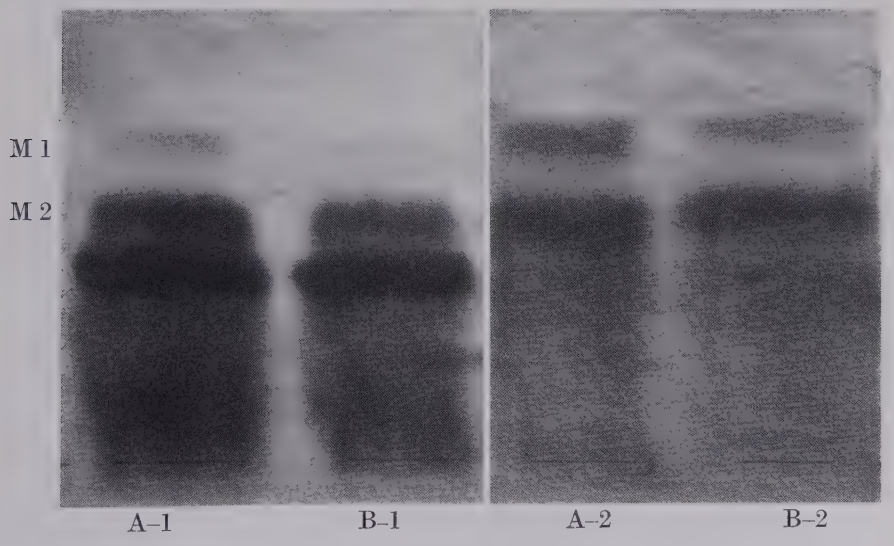


FIG. 6. Paper electrophoretic patterns obtained in a patient with rheumatoid arthritis before (*A*) and during (*B*) treatment with cortisone. *A*-1 and *B*-1 are stained for protein and *A*-2 and *B*-2 for carbohydrate. It should be noted that the carbohydrate stains the albumin region only faintly. A decrease in both M 1 and M 2 is observed.

electrophoresis at pH 4.5 followed by PAS staining of the serum-bound carbohydrates and they reported elevated values for the M1 fraction. Rheumatoid sera were studied in citrate phosphate buffer at pH 4.5 in paper electrophoresis experiments. In a certain number of cases they found electrophoretic heterogeneity of the M1 fraction (Laurell 1951). The M1 fraction is probably identical with acid alpha-1 glycoprotein (Schmid, 1953), or orosomucoid (Winzler *et al.*, 1948). Goodman, Ramsey, Simpson, and Brennan (1957) have described methods for its determination with chicken antiorosomucoid sera.

Patterns obtained in paper electrophoresis at pH 4.5 with "normal" and rheumatoid sera are shown in Fig. 5. The strips to the right have been stained for carbohydrate (PAS stain) and those to the left for proteins. The M1 and M2 fractions are readily observed in the PAS strip from rheumatoid arthritis. It should be noted that the normal serum has only a little PAS staining in the M2 fraction. Electrophoretic paper strips from a case with rheumatoid arthritis before and during the administration of cortisone are shown in Fig. 6. It is noted that the M1 and M2 fractions decrease. Paper electrophoresis of sera at pH 4.5 has also recently been studied by Schultze and Biel (1955), Heremans (1956), Markham *et al.* (1956), Sonnet (1956), and Bodman (1958). Sonnet (1956) discussed possible connections between M2 and haptoglobin.

The present study deals with methodological problems encountered during work with the vertical zone electrophoresis apparatus. The aim has been to develop methods and procedures to make possible the isolation of serum fractions such as M1 and M2, albumin, and gamma globulin, from individual sera in amounts which are sufficient for a chemical and physico-chemical characterization. Only if this is achieved may the changes reported in the above preliminary studies be analyzed further.

There are reasons to believe that at least some of the recently reported microheterogeneities of purified proteins may be due to genetically controlled modifications of the molecular structure (Bennhold, Peters and Roth, 1954; Smithies and Walker 1956; Huisman, 1957; Poulik, 1957; and Knedel, 1958).

ZONE ELECTROPHORESIS—A GENERAL DISCUSSION

It is convenient to distinguish between the boundary and zone methods in electrophoresis. In boundary electrophoresis the migration of a boundary separating two solutions is studied by optical or analytical methods. In zone electrophoresis a zone of a sample with components of different mobilities splits up into completely separate bands. The components obtained have to be stabilized against the influence of gravity in order to move as well-defined zones in the electric field. Usually they have higher densities than the surrounding solution and this causes convection if a stabilizing medium is not introduced.

Agar gel, which may be permeable to protein solutions, was used as a stabilizing medium in the early experiments of Field and Teague (1907) to study the migration of diphtheria toxin and antitoxin in the electric field. Coolidge (1939) used ground glass wool as convection stabilizer in zone electrophoresis of serum proteins. He also describes the use of sucrose or starch added to the buffer in the lower reservoir to obtain good layering of the protein zone and he may thus be said to have used a density gradient in vertical zone electrophoresis. Convection is not entirely abolished through the influence of the stabilizing medium but it is prevented from spreading along the column, nevertheless microconvections must certainly be present. Among other methods for counterbalancing the gravity influence the density gradient column may be mentioned (Philpot, 1940; Kolin, 1954 and 1958; Svensson and Valmet, 1955; Svensson, Hagdahl, and Lerner, 1957; Berg and Beeler, 1958).

Zone electrophoresis has been the subject of several reviews and monographs in the last few years, e.g. McDonald (1955). A selection of references to books or articles of more general interest is given by Tiselius (1957), Laurell (1957), and Durrum (1958). Recent developments in this Institute have been summarized by Porath (1957*a*) and Tiselius (1958).

The differential migration observed in boundary or zone electrophoresis depends on the differences in electrophoretic mobilities of the ionic con-

stituents in the solution. A full treatment of the theory of zone migration in stabilized media lies outside the scope of this article. The following brief treatment only aims at giving a general indication of the nature of the physical ideas involved.

It is convenient to start the discussion with the qualitative changes which occur at a moving boundary in free electrophoresis and to consider the simplest system possible (MacInnes and Longsworth, 1932).

In one limb of an U-tube a sharp boundary is formed between 0.1 *N* potassium iodate and 0.1 *N* potassium chloride, the potassium chloride being on top of the denser potassium iodate solution (Fig. 7). One requisite for a sharp moving boundary to be retained when the current passes through the column is that the anode electrode is connected to the potassium chloride solution since the chloride ion has a higher mobility than has the iodate ion. We assume further that a constant potential difference is maintained between the two ends of the U-tube.

It will be recalled that ion mobilities are velocities developed by the ions in the solution under the influence of a unit field; that the transference number of an ion is the fraction of the total current carried by the ion in the solution, and that the specific conductivity is the reciprocal of the specific resistance. The conductivity of the solution is thus a measure of the quantity of electricity transferred across a unit area per unit potential gradient per unit time.

The 0.1 *N* potassium chloride solution has a higher conductivity than the potassium iodate solution and thus the potential gradient in the two solutions will be different at the moment when the electric field is applied. In the boundary region between the two solutions (Fig. 7) there will momentarily be a tendency for more chloride ions to move out of this region than for iodate ions to move into it.

Since electro-neutrality must prevail, the conductivity of the boundary region diminishes and therefore the electric field in the boundary region increases at the same time as the electric field in the two solutions above and below the boundary region slightly decreases. The IO_3^- ions in the boundary region will thus be in a higher electric field than before and their velocity thus increases. Because both sides of the moving boundary must obviously move with the same velocity it follows that the mobility of the chloride ion times the electric field in the 0.1 *N* KCl solution must be equal to the mobility of the IO_3^- ions times the electric field in the adjusted solution (0.06 *N* IO_3^- in Fig. 7). Also it can easily be shown that the concentration in the adjusted IO_3^- solution is related to the Cl^- concentration in the same way as the transference numbers for Cl^- and IO_3^-, respectively.

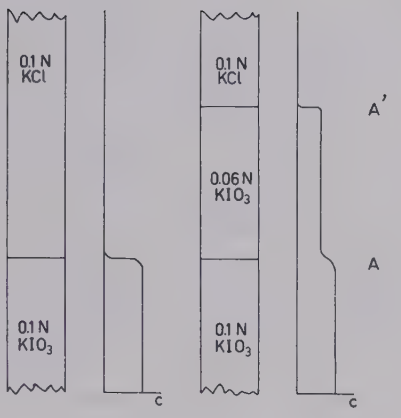


Fig. 7. A moving boundary experiment with 0.1 N KCl on top of the 0.1 N KIO_3 solution. Modified after MacInnes and Longsworth (1932).

To the left the initial situation before the electric potential is applied. To the right the boundary originally found at A has moved to A'. The concentration (c) of the indicator ion, iodate, is shown in the small diagrams. The potential gradient in the region A–A' is increased to give the IO_3^- ion the same velocity as the Cl^- ion at the boundary A'.

At the position of the starting boundary between the two solutions (A, Fig. 7) there will remain a boundary separating the original 0.1 N KIO_3 solution from the adjusted 0.06 N IO_3 solution (MacInnes and Longsworth, 1932). This boundary will remain stationary if the IO_3^- mobility is independent of concentration. Diffusion, however, takes place at this boundary as shown in Fig. 7. At the moving boundary A' in Fig. 7 diffusion is however counteracted by electric field effects. It can easily be shown that an IO_3^- ion, which through diffusion should come into the Cl^- region, is slowed down because of the low potential gradient in this region. The same type of argument applies to a Cl^- ion which accidentally through diffusion should pass into the adjusted IO_3^- solution. Below A' (Fig. 7) this Cl^- ion is in a higher potential gradient than before, and as its mobility is higher than the IO_3^- ions it will soon move out of this region. An interesting result, although self-evident, is that the diffusion may be counterbalanced if a suitable electric field is applied. However, experimental conditions often limit this possibility because of convection caused by heat generated when the current passes through the column. In the above-mentioned discussion only the anions have been dealt with, but as the solution in the different concentration areas must be electrically neutral the cation K^+ must equal the concentration of the anions in the different solutions. The stationary boundary at A is of the same type as that encountered in free electrophoresis at the ε-boundary. At the δ-boundary in free electrophoresis both the protein components and the buffer salts change their concentration. Already from the above superficial discussion it is evident that in a more complex system than that discussed above ions present on both sides of a moving or stationary boundary have to change their concentrations at this boundary. The

changes in potential gradient across a boundary must naturally affect all ions in the same way.[1]

The sharpening effect present in a moving boundary is indicated at A' in Fig. 7 and it should be recalled that this sharpening effect can only take place if the current is passed in such a direction that the ion with the higher mobility moves ahead of the ion with lower.

Kendall (1942) summarized a series of experiments which had been performed by his group during the past twenty years. They were trying to separate chloride isotopes by means of zone electrophoresis and although they did not achieve the goal, their presentation anticipates many features of contemporary zone electrophoresis. They used agar gel as a stabilizing medium and a zone of sodium chloride (Fig. 8) was placed between agar gel containing sodium acetate and sodium hydroxide, respectively, in a long horizontal pyrex tube. The current direction was such as to move the chloride ion towards the sodium hydroxide as shown in Fig. 8. In this way both the front and rear boundaries of the chloride zone were subjected to the sharpening effect described above.

Kendall realised that, in order to maintain the width of the chloride section constant, the concentrations of the various salts in their respective gels had to be arranged according to the transference numbers of their anions. He states that, "Even if this is not done exactly, however, the boundary concentrations soon automatically adjust themselves to the required ratios". Thus as soon as this adjusted concentration has been achieved the width of the zone remains constant throughout the experiment. When the front of the chloride boundary had almost reached the end of the tube the apparatus was taken apart and the two rear sections were discarded. Two new sections filled with sodium hydroxide gel were inserted in front of the chloride and two new electrodes were fitted as before. The chloride zone was then allowed to migrate into these new sections and Kendall described how the whole procedure could be repeated until the chloride zone had progressed through about 100 feet of the gel. The effective migration distance thus achieved as early as 1942 has probably not yet been surpassed in spite of modern developments which utilize migration against a hydrostatic flow. He achieved, in test experiments with iodide and chloride ions, results which show that a very considerable degree of separation was obtained even if the differences in mobility were less than 1 per cent. In the molten state differences in mobility of the isotopes may more easily be detected

[1] It is of historical interest to note that Faraday (1833) described a method for forming a sharp boundary between two solutions in order to study changes taking place when a current was passed through the solution.

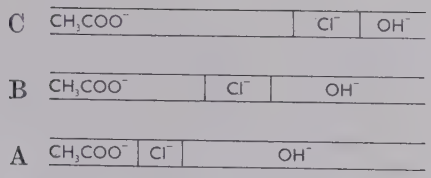


FIG. 8. A migrating chloride zone in agar gel (Kendall, 1942).

A. The initial state where the chloride zone is surrounded by agar gel with acetate to the left and agar gel with sodium hydroxide to the right.

B. The width of the chloride zone is adjusted when the zone migrates. The initial expansion should be noted. The concentration in the chloride zone is thus automatically adjusted in accordance with the transference numbers of the three ions CH_3COO^-, Cl^-, and OH^-.

C. A later stage where the chloride zone moves with constant velocity and constant width.

due to the absence of hydration, which must surely tend to decrease such differences and this may be the reason why later work with electrophoretic migration of the ions was successful (Klemm and Monse, 1957; Chemla, 1958). The fact that the width of the chloride zone automatically adjusted itself so that the concentrations of the chloride, the acetate, and the hydroxide ions were in accordance with their transference numbers is shown diagrammatically in Fig. 8. This effect is especially clear in the special case where both boundaries surrounding a zone are being automatically sharpened.

In zone electrophoresis with proteins the conditions prevailing are more complicated. Some information may however be gained from studies obtained in free electrophoresis and Fig. 9 illustrates some of the changes taking place during the early phase of the separation of two protein components. The diagrams are based on the calculations of Longsworth (1947) which were also used by Alberty (1953) in discussing the changes which take place in the free electrophoresis of a solution of two proteins of the same concentration but with different mobilities.

In Fig. 9*A* the initial state is shown when the two proteins are located in the same zone and the concentration of one is twice that of the other. The Donnan equilibrium which is obtained during dialysis against the barbiturate buffer gives rise to a decreased concentration of barbiturate ion in the protein zone and hence the pH in this zone is also slightly lower than in the surrounding buffer. When the voltage is applied the potential gradient in the protein zone is slightly greater than in the buffer column.[1]

[1] Lagercrantz (1944) reported direct conductivity measurements in moving boundary experiments.

After the separation has started, the changes taking place in the buffer are shown in Fig. 9 *B*. It should be noted that the barbiturate concentration increases in the region where the protein zone was originally applied and that successive changes in the barbiturate concentration take place at different levels in the column during this initial stage of the separation. At the same time changes are observed both in the potential gradient and in the pH in the solutions. When the separation has proceeded further and the two components have completely separated from each other, a certain sharpening effect will take place at the front boundary if the forces suppressing the diffusion are not prevented by the pH changes taking place at the boundary (Alberty, 1953). If the protein zone has been dialyzed against buffer, a slight expansion of the zone takes place, and this expansion will be more marked for the component of low rather than the one of high mobility. The conductivity in the peak of a protein solution is, as a rule, lower than in the surrounding buffer solution, as found in experiments.

A theoretical treatment of ion migration at moving boundaries is found in papers by Kohlrausch, Weber, von Laue, Tiselius, Longsworth, Dole, and Svensson. References to these papers are given by Alberty (1953). The migration of zones in stabilized media under various conditions has been discussed by de Wael and Wegelin (1952), Spiegler and Coryell (1952), Weber (1953), Klemm and Monse (1957), Pohlit and Schittko (1958), and Edward (1958).

In free electrophoresis there is often a need for diminishing or abolishing the δ- and ε-boundaries, especially when long runs are made, since during compensation the δ-boundary may cause disturbances in the descending side. Methods for minimizing these boundaries have been described by Hacker (1933), Longsworth and MacInnes (1940), Svensson (1946), Wiedemann (1947), and Hoch (1950).

A feature of vertical zone electrophoresis which seems to have been neglected so far is its use for the adjustment of the concentration of a solution in order to suppress δ- and ε- boundaries in a subsequent moving boundary experiment. This may easily be done with a pure substance if a broad zone of the substance under investigation is dissolved in the buffer which is to be used in the moving boundary experiment. This is fed in to the long vertical column from below and zone electrophoresis is allowed to take place for a time sufficient to attain this adjusted concentration. The broad zone may now be eluted and material from its center together with the original buffer used directly in a moving boundary experiment without any further handling. This technique may be useful for obtaining mobility data particularly for low molecular weight substances since an equilibrium

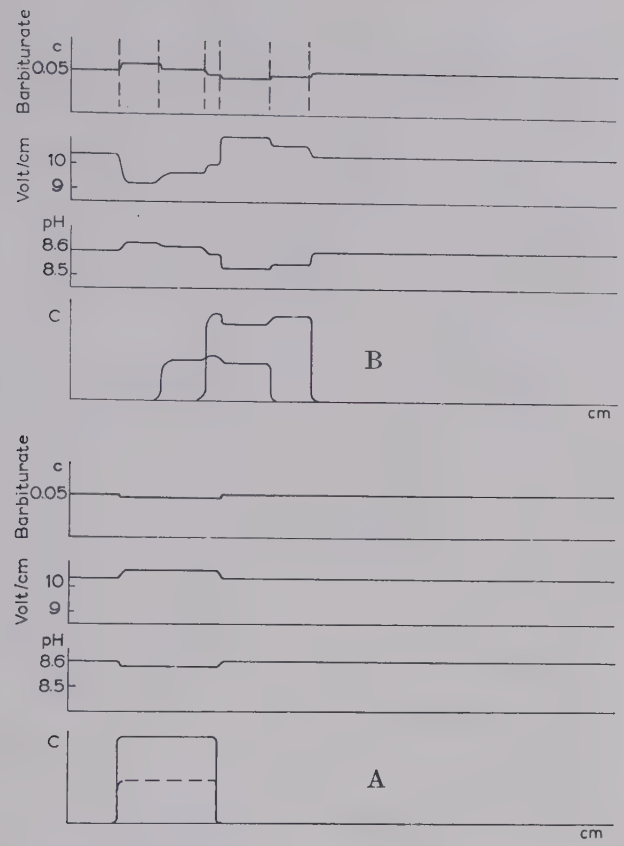


FIG. 9. A diagrammatic representation of changes taking place in vertical zone electrophoresis based on calculations by Longsworth (1947).

A. The initial state with the two components. Protein concentration (C), pH, and barbiturate concentration (c) is shown. The protein zone consisting of two components in ratio 1:2 is dialyzed against the barbiturate buffer and the changes in pH and in barbiturate concentration are dependent on the Donnan equilibrium obtained during the dialysis. At the moment when the field is applied the field strength will be slightly higher in the protein zone.

B. Changes in the column during the first stage of migration. A pH gradient, a field gradient, and a change in barbiturate concentration remain in the region where the protein zone started to migrate. In a later stage not shown in the figure the components are completely separated. A slight pH decrease and an increased field strength over the protein zones are then obtained.

with buffer ions cannot be obtained for these materials through the process of dialysis.

Because of geometrical reasons equipotential surfaces in a long horizontal vertical zone electrophoresis apparatus must be, in the first approximation,

cross-sections of the column. It is readily apparent that at the bottom and the top of the column the equipotential surfaces may be slightly curved depending on the geometrical form of the electrodes and the dimensions of the buffer reservoirs above and below the column. However, these equipotential surfaces must be almost perpendicular to the axis of the column. As the temperature (Porath, 1957 *b*) in the column is naturally lower near the cooled surfaces, the conductivity of the solution must be greater in those parts of the column which are not efficiently cooled. Since the specific conductance of an aqueous solution increases approximately two per cent per degree (Bull, 1951), the conductance within different regions of a cross-section of the column has different values depending on the prevailing temperature. The current density in different elements in the cross-sections has to be adjusted so as to give nearly parallel equipotential surfaces. This is only true if the temperature is not allowed to increase to such an extent that severe heat convections disturb the stabilizing medium.

To apply the protein zone to the column from below may have certain advantages.

1. The direction of the current may be arranged so as to allow the component of interest to be eluted first and thus losses due to adsorption in the column are minimized.

2. A slight hydrostatic pressure under the separation column may be used to displace the zone upwards against the direction of migration of the components of interest. This pressure is obtained through the action of the buffer mixing pump, Fig. 35, but not more than a difference of 3 or 4 cm in the level of the two electrode vessels should be employed because there is a danger for the stabilizing medium to be disturbed.

Migration against the buffer flow was used in moving boundary experiments by Tiselius (1937) and this principle was also used in zone electrophoresis by Brewer, Madorsky and Westhaver (1946), and Porath (1956).

Electric field effects will stabilize to a certain extent the lower boundary of the protein zone since diffusion at this position is slightly counterbalanced and the perpendicular equipotential surfaces in the upper part of the column may tend to counteract a spreading at this boundary of microconvections of the type described in Fig. 32. Because the upper boundary of the protein zone is stabilized by a density gradient due to the difference in density between the protein zone and its adjacent buffer zone with which it has been equilibrated during the dialysis, the equipotential surfaces in the protein and buffer zones will be perpendicular to the axis of the column.

Provided the protein zone has been dialyzed against the buffer used in

the experiment, the ratio protein ion to buffer ion in this zone will determine the extent of the initial expansion of the zone. This is one of the reasons why minor serum components in starch gel electrophoresis give smaller bands than does the albumin fraction. This expansion of the albumin fraction is also observed in experiments with tris-buffer or tris-EDTA buffer (p. 54) as well as in guanine buffers. This expansion is also clearly demonstrated in the cellulose acetate strips used by Kohn (1957). In paper electrophoresis in a moist chamber this expansion of the albumin band is counteracted by conductivity gradients in the paper caused by the evaporation of the buffer solvent.

The possibility of using field gradients for sharpening the zone is discussed by Haglund and Tiselius (1950) and has been systematically used by Porath (1956). Zone sharpening, by means of adjacent zones with low conductivity may not, however, be as useful for protein zones as for low molecular weight substances, because of the pH and heat gradients developed in these zones. These gradients may produce asymmetrical peaks which may simulate two components instead of giving a symmetrical peak which should otherwise have been obtained for a single component.

CONSTRUCTIONS

Vertical Zone Electrophoresis Apparatus

The apparatus described is a modification of the Porath-Tiselius vertical zone electrophoresis apparatus (Haglund and Tiselius, 1950; Flodin and Porath, 1954; Porath, 1957*b*), in which a more efficient cooling system has been introduced. The author is indebted to Dr. J. Porath and Mr. S. Jerstedt of the Institute of Biochemistry, Uppsala, for helpful advice in connection with previous models and in construction of the present apparatus. Grateful acknowledgement is also due to Mr. L. Matsson, head of the Institute's workshop, for his untiring interest and expert assistance in working out many constructional details.

The apparatus (Fig. 10) consists of a separation-column (*C*) filled with supporting medium, an inlet head (*D*) for central cooling, a top piece (*E*) and a connecting ring (*F*) which serves as a sliding airtight joint to the vertical buffer column (*G*). The electrode vessels (*I*) are placed beneath the two columns. The apparatus rests on a polyvinylchloride shelf (*K*) which is screwed to an aluminium support. This, in turn, is anchored by two strong, pliable cotton belts with safety buckles (Fig. 11) to a stainless steel pipe (*L*) which rests on the floor. The electrodes (*J*, Fig. 10) are held in two holes in the supporting shelf. The guiding bracket (*N*) is provided with inlet and outlet tubes for the cooling water and it also supports the syringe device whereby the protein zone is applied to the separation column from above. Pyrex glass has a higher heat conductivity than perspex and is therefore used for all cooling tubes and to separate the supporting medium from the outer cooling jacket in the separating column. Perspex is used for the outer wall of the cooling jacket, the buffer column (*G*), the inlet head (*D*), and the top piece (*E*).

Although the construction is essentially straightforward a few points may be mentioned. It is advisable to select glass tubes of proper dimensions for the cooling tubes and for the walls of the separation column before fittings and collars are made. The dimensions of the separation column can then be adjusted to the tubes. All tubes should be selected at least in duplicate so that spare parts are readily available. As the diameter of a glass tube

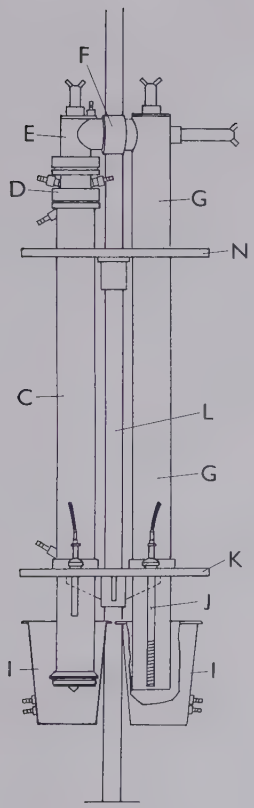


FIG. 10. A schematic drawing of the main parts of the zone electrophoresis apparatus. The separation column (*C*) which is packed with the stabilizing medium and the buffer column (*G*) are both supported by the shelf (*K*). Inlet head of the central cooling (*D*), top piece (*E*), connecting ring (*F*), guiding bracket (*N*), electrode vessels (*I*), electrodes (*J*), stainless steel pipe (*L*).

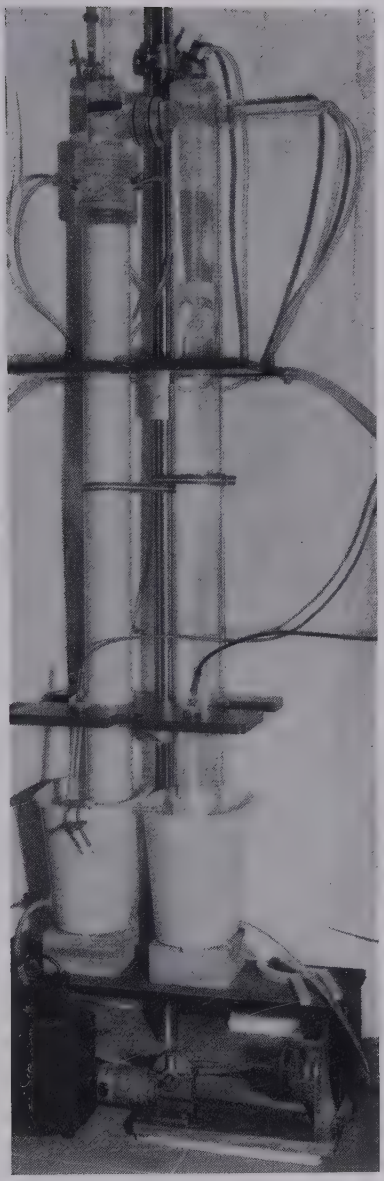


FIG. 11. Zone electrophoresis apparatus with the electrode vessels in place under the two columns. In front of the apparatus is the buffer pump.

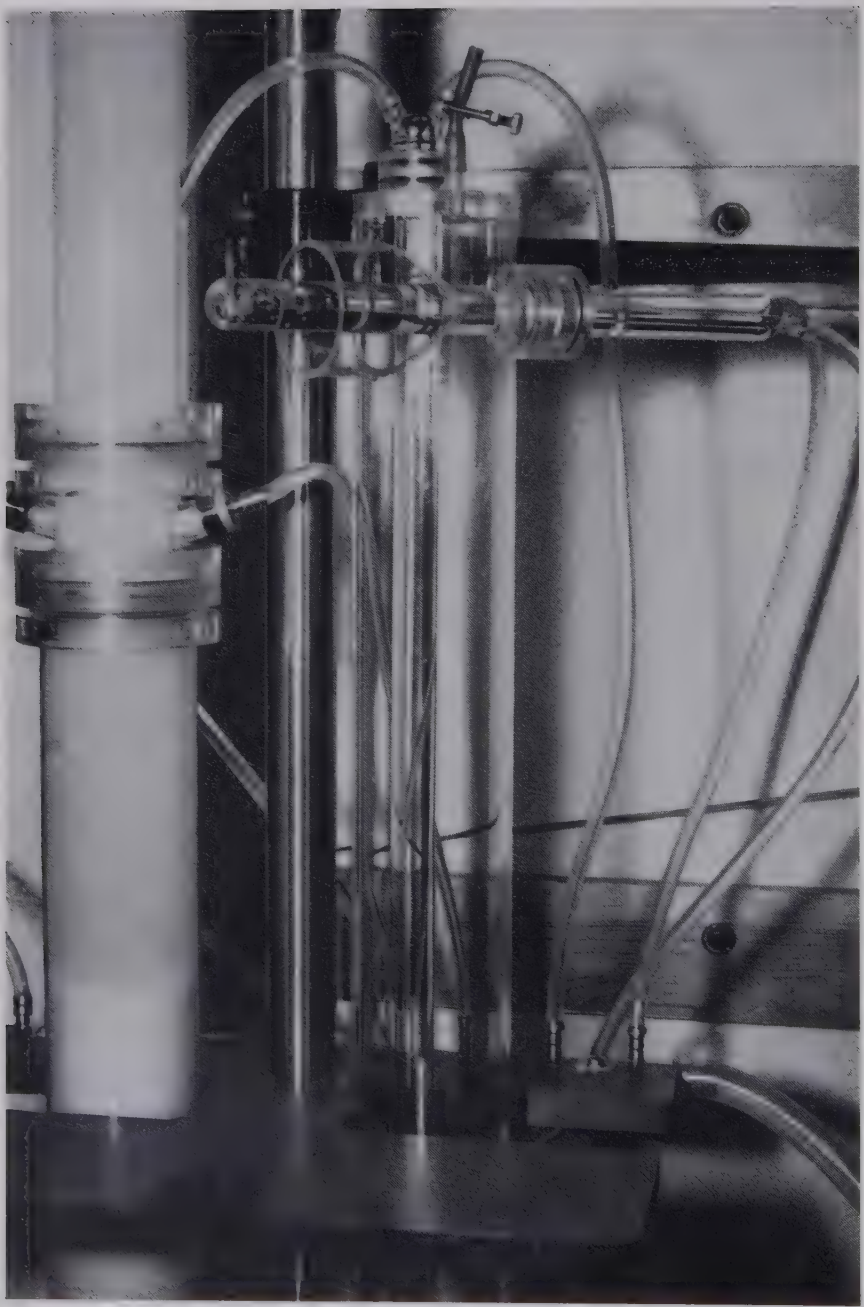


FIG. 12. The upper part of the zone electrophoresis apparatus during the packing procedure. The top piece has been removed and replaced by a packing tube. The horizontal cooling tube in the buffer column is shown in the figure.

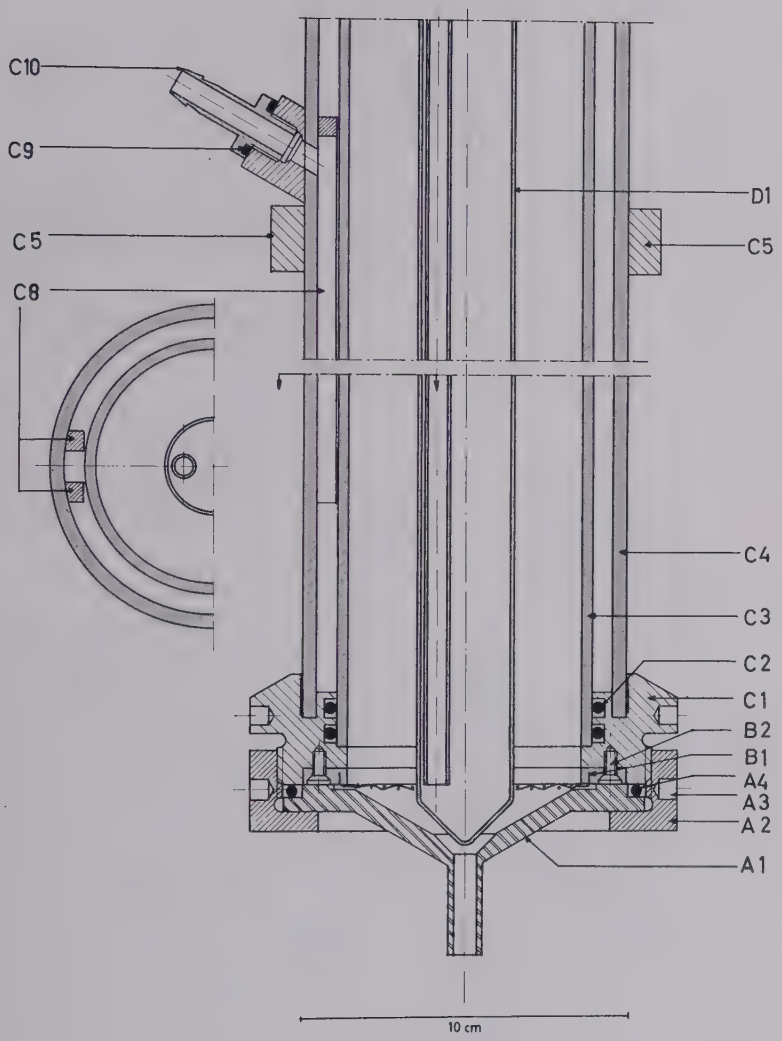


FIG. 13. Construction drawing of the lower part of the separation column (*C*, Fig. 10). The supporting medium is placed in the annular space between *D*1 and *C*3. The attached funnel (*A*1) is used during elution of the zones. The central cooling tube (*D*1) as well as the outer wall of the separation compartment (*C*3) are made of pyrex glass. *C*3 and *C*4 form walls in the outer cooling jacket. The inlet for the outer cooling jacket is shown at *C*10.

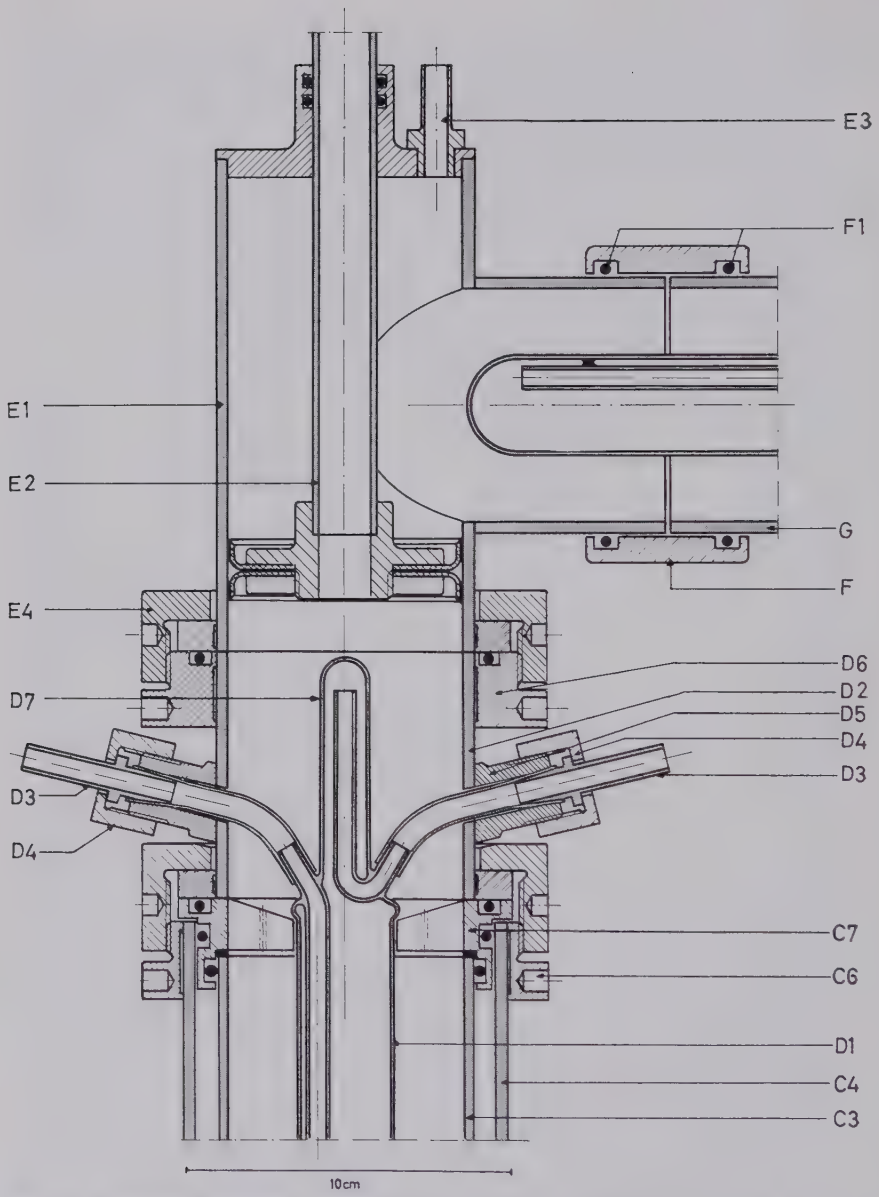


FIG. 14. Construction drawing of the upper part of the separation column (*C*, Fig. 10), the inlet head (*D*, Fig. 10) of the central cooling tube and the top piece (*E*). The perspex piece *C*7 which supports the central cooling tube is also shown in Fig. 22 and the inlet head of the central cooling tube in Fig. 21. As described in the text, a second central cooling tube may replace the piston (*E*2) and the upper part of the central cooling tube (*D*7).

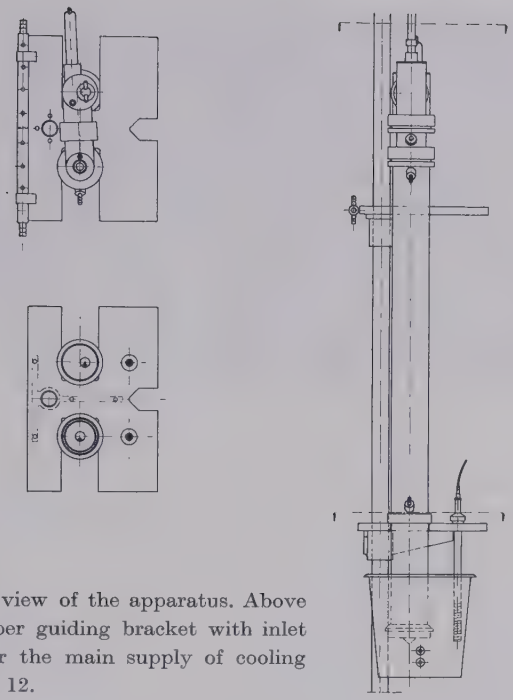


FIG. 15. Side view of the apparatus. Above right, the upper guiding bracket with inlet and outlet for the main supply of cooling water, cf. Fig. 12.

frequently varies, it is recommended that both ends of the tube (*C*3, Fig. 13), be ground in a lathe to insure an even pressure on the O-rings. These rings, which seal the cooling water from the buffer, are made of perbunan, an oil-resistant synthetic rubber.[1] It is important that there should be no leakage between the cooling water and the buffer. Narrow passages have been avoided in the construction as the uncontrolled heat production and appreciable potential gradient resulting would diminish the potential gradient in the separation column and reduce its efficiency. The height of the supporting shelf is adjusted to leave enough room under the separation column for a fraction collector when the electrode vessel is removed.

The outer water jacket (*C*4, Figs. 13 and 14) of the separation column and the two threaded collars (*C*1, Fig. 13 and *C*6, Fig. 14) are made of perspex. These are sealed to the perspex tube (*C*4) as is also the supporting ring (*C*5, Fig. 13). A threaded metal nipple (*C*10, Fig. 13) with a perbunan O-ring seal (*C*9, Fig. 13) serves as inlet for the cooling water. Two small perspex guides (*C*8, Fig. 13) direct the stream of water to the bottom of the column. The outlet for the cooling water is shown in Fig. 15. A thick filter

[1] Simrit-Werk, Weinheim, Germany.

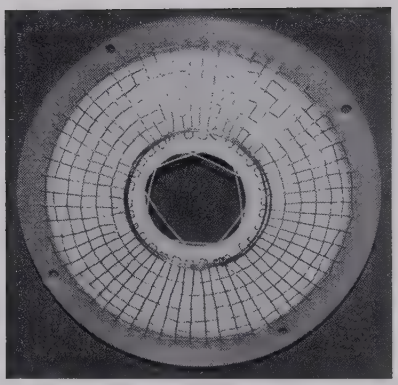


FIG. 16. The bottom plate of the separation column, made of a polyethylene ring (*B*1, Fig. 13) and a platinum net, supports a thick filter paper disc. It has a hole for the central cooling tube. After the platinum net has been placed on the tool (Fig. 18) electrical spot-welding is employed to secure the formed net. Spun nylon fishing line helps to ensure a tight fit between the platinum net and the central cooling tube.

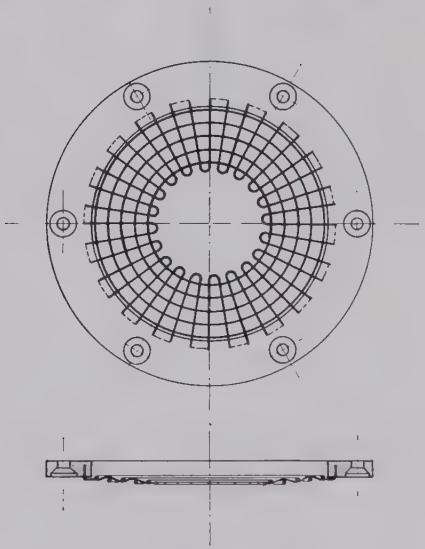


FIG. 17. Construction drawing of the platinum net for the bottom plate.

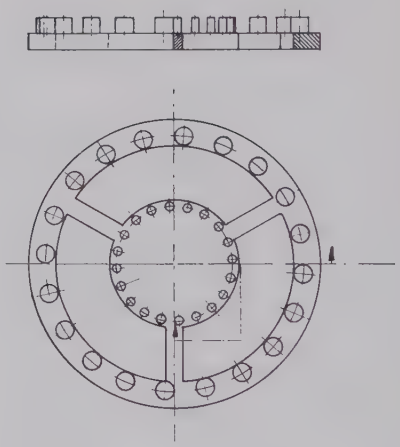


FIG. 18. Tool used for construction of the platinum net (Fig. 17).

paper disc, perforated to fit the central cooling tube (*D*1, Fig. 13), is placed on a supporting net made of platinum wire (Fig. 16). The use of platinum was suggested by Dr. Porath. The net (Fig. 17) is made with a special tool shown in Fig. 18, and is heat-sealed into a ring (*B*1, Fig. 13) made of polyethylene or polyvinyl chloride. The net and the ring are shown in Figs. 13 and 16. Screws (*B*2) made of a strong fiber material anchor the net to the collar (*C*1, Fig. 13). Spun nylon fishing line (Fig. 16) helps to ensure a tight fit between the platinum net and the central cooling tube (*D*1). The

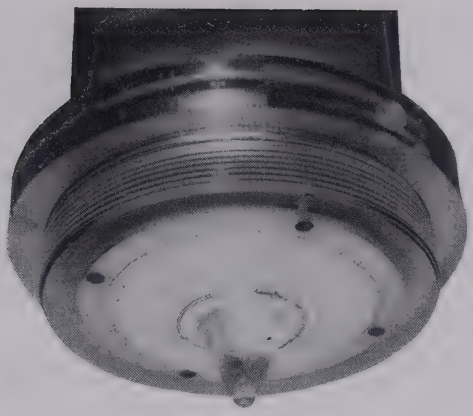


FIG. 19. View of the lower end of the separation column as it is immersed in the electrode vessel during the run. The bottom funnel (*A*1, Fig. 13) has been removed. The protruding lower end of the central cooling tube is shown.

nylon net is secured by a knot which is slightly melted in a micro flame to prevent slipping. The diameter of the lower end of the central cooling tube (*D*1) diminishes slightly when passing through the bottom plate. The area of the thick filter paper on the bottom plate is thus greater than the cross-sectional area of the separation column, and the protruding central cooling tube stretches the nylon threads slightly in the bottom plate and gives an efficient cooling of this area (Fig. 19). Experience in this Institute has shown the necessity of constructing the bottom plate so as to minimize the possibility of bubbles adhering to it. The protruding end of the central cooling tube (*D*1) gives rise to convectional stirring under the bottom plate.

During elution a special perspex funnel (*A*1, Fig. 13) is attached to the separation column by means of a threaded collar (*A*2). A water-tight seal is formed by the O-ring (*A*4). A pair of wrenches, shown in Fig. 20, are used

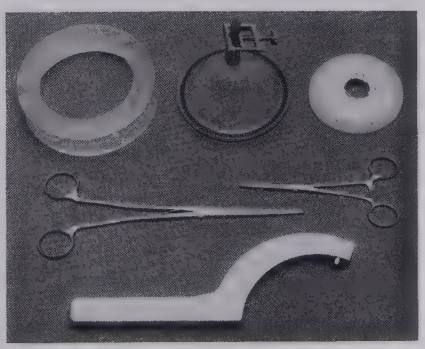


FIG. 20. The bottom funnel (*A*1, Fig. 13) with attached rubber tubing and hose-cock and the threaded perspex collar (*A*2, Fig. 13) are shown in the top row. Below, a wrench used for tightening the perspex collar and the forceps mentioned in the text.

for tightening the threaded collars in the apparatus. Rubber tubing with a castaloy hosecock (Fig. 20) is attached to the nipples of *A*1.

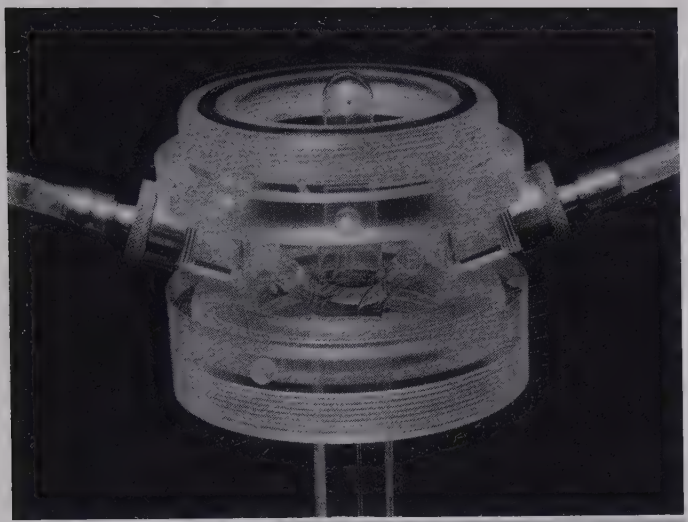
Two O-rings plus a special perspex piece (*C*7, Figs. 14 and 22) seal the upper portion of the outer cooling jacket and support the weight of the inner cooling tube (*D*1) which rests on three protruding lugs. Through holes in these lugs three needles may be inserted when the zone is applied to the column.

The inlet head of the separation column (Fig. 21) is made of perspex and has threaded collars by which it can be attached to the separation column (*C*, Fig. 10) and to the top piece (*E*, Fig. 10). Short polyvinylchloride tubes are attached to the central cooling tube (*D*1). The tubing is secured by strong spun nylon threads (Fig. 21), the knots of which are melted in a micro flame. Two conical metal nipples (*D*3, Fig. 14) connect the tubing to the inlet and outlet of the cooling water. By widening the tubing over the slightly heated nipples a close fit is secured. A metal collar (*D*4, Fig. 14) is screwed to the perspex piece (*D*5, Fig. 14), which forms a watertight seal with *D*3. The same principle applies here as in the plastic tubing adaptors[1] used for the application of the zones.

Rubber tubing and a hosecock are attached to the nipple *E*3 (Fig. 14). The connecting ring (*F*, Figs. 10 and 11) joins the separation column with the buffer column, and two O-rings (*F*1, Fig. 14) form a water-tight seal. The top piece (*E*, Fig. 10) is screwed by a threaded collar (*E*4, Fig. 14) to the inlet head (*C*, Fig. 10) of the separation column.

The upper part of the long buffer column (*G*) is shown in Fig. 23. It is made of perspex and has a nipple (*G*2) with attached rubber tubing and hosecock. A sliding perspex joint (*G*3), sealed with O-rings (*G*4), guides the horizontal pyrex cooling tube (*G*5). When the piston (*E*2, Fig. 14) is removed to the upper recess in the top piece (*E*), the horizontal tube (*G*5) can be moved forward into the space formerly occupied by the piston, thus cooling the buffer efficiently. A long vertical pyrex tube (*G*8, Fig. 23), inserted through a perspex joint (*G6*, Fig. 23) with two O-rings (*G*7, Fig. 23), serves to cool the buffer in the buffer column. The lower part of the long pyrex tube is secured by a supporting ring located at the bottom of the inside of the long buffer column (*G*1, Fig. 23). When the separation column is packed with the supporting medium the top piece (*E*) is removed and a long packing tube (*H*1, Figs. 24 and 29) is screwed to the inlet head (*E*) of the separation column by means of a threaded collar (*H*2, Fig. 24). This tube is made of perspex and the lower end has an attached perspex

[1] Clay-Adams, Inc., New York 10, N.Y., U.S.A.



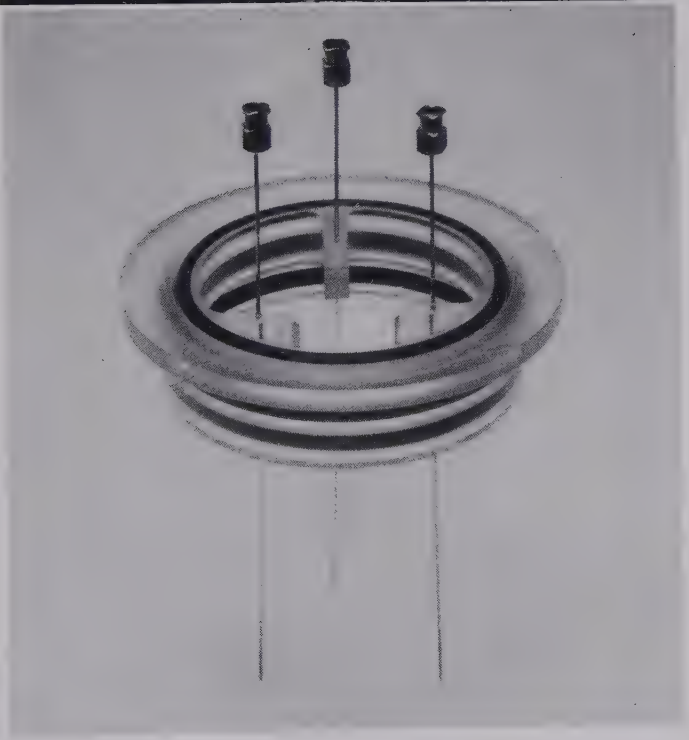


FIG. 21. The inlet head of the separation column with the central cooling tube. Note the short polyvinyl tubings which are secured by strong spun nylon threads.

FIG. 22. The perspex piece (*C*7, Fig. 14) which together with two O-rings forms the upper seal in the outer cooling jacket of the separation column. Three lugs support the central cooling tube (*D*1, Fig. 13). The lugs have polyethylene lined holes for three needles which are used when the protein zone is applied to the column from above.

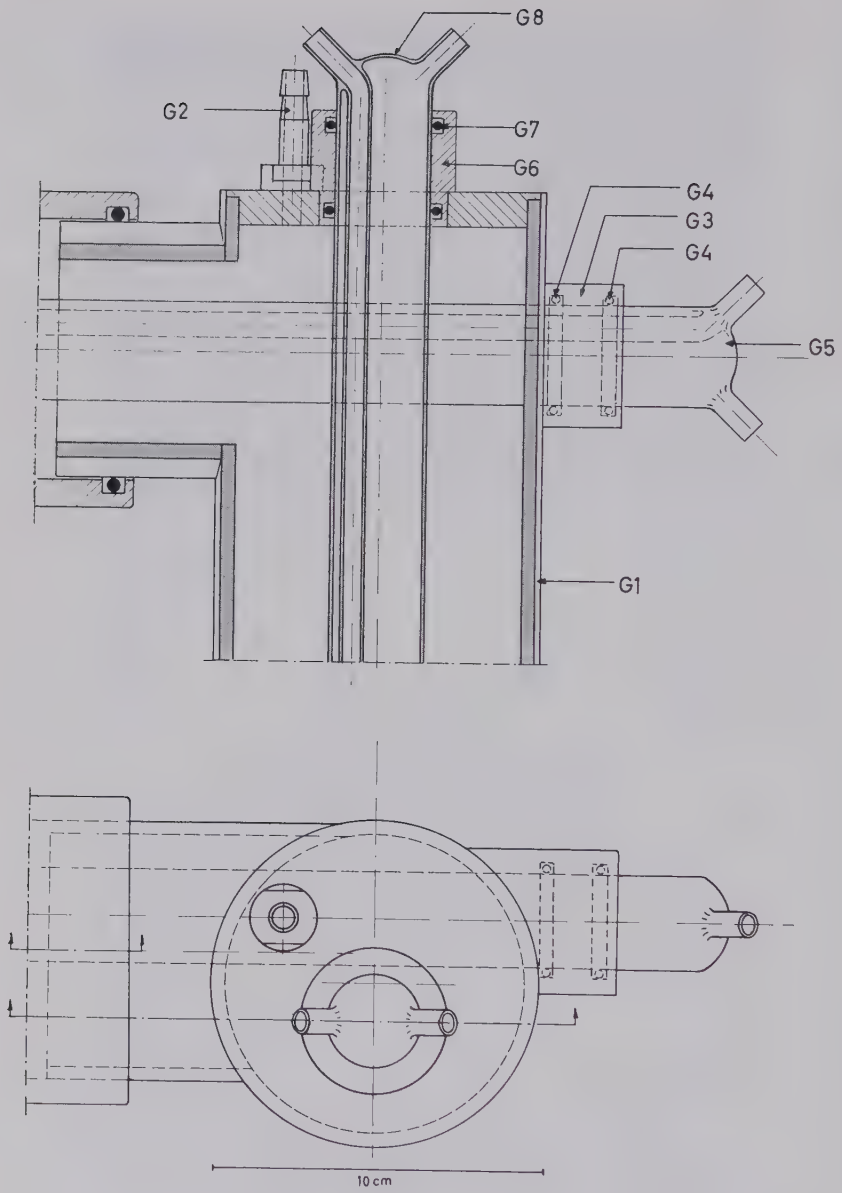


Fig. 23. Construction drawing of the upper part of the buffer column (*G*, Fig. 10). Details of this piece are also shown in Fig. 12. The length of the buffer column *G*1 is 160 cm.

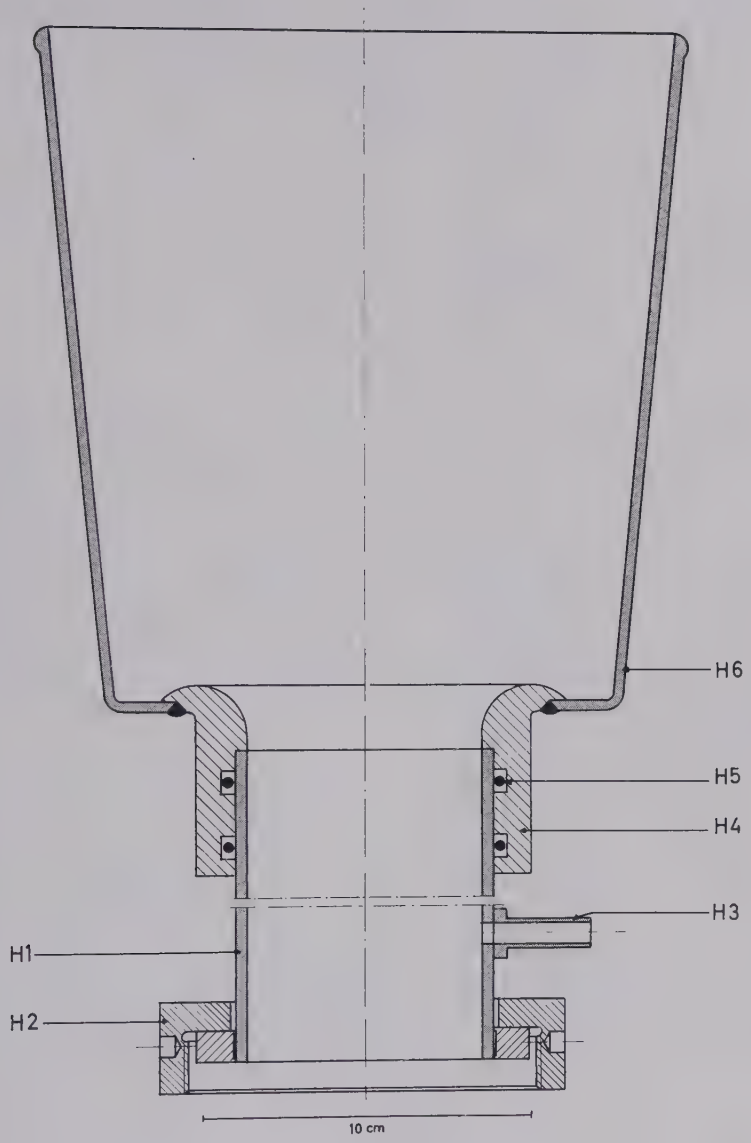


FIG. 24. Construction drawing of the packing tube, cf. Fig. 29.

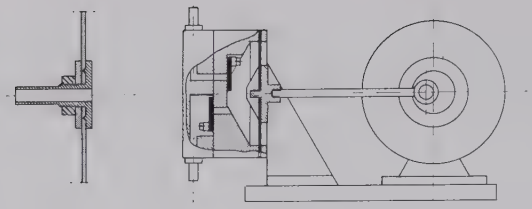


FIG. 25. Nipples which are attached to the commercial polyethylene waste-baskets used as electrode vessels.

FIG. 26. Schematic drawing of the buffer pump. The piston is moved by a variable eccentric.

nipple with rubber tubing and hosecock (*H*3, Fig. 24). A polyethylene ring (*H*4, Fig. 24) with two O-rings (*H*5) forms a seal against the perspex tube (*H*1) and is fixed by hot gas welding to a commercial plastic bucket (*H*6) (Kinney, 1957). The electrode vessels, made from commercial plastic waste-baskets and equipped with two nipples (Fig. 25), are connected with the pump which serves to mix the buffers at the anode and cathode, thus preventing pH changes during the run. The nipples are made from polyethylene and fastened by a threaded polyvinyl ring shown in Fig. 25. The pump is shown in Fig. 26. All parts of it which come in contact with the buffer are made of perspex or synthetic perbunan rubber. The piston is moved by a variable eccentric.

The zone electrophoresis apparatus may be simplified without loss in performance if the piston (*E*2, Fig. 14) is replaced by a cooling tube. The collar (*D*6, Fig. 14) may be removed and a connection with a simplified top piece made instead by sliding O-rings. To replace the function of the piston (*E*2) a removable bottom piece (*M*1, Fig. 27) for the buffer column (*G*1) is made from perspex. Two O-rings (*M*2, Fig. 27) form a tight connection to the bottom of the buffer column (*G*1) and the column may be filled through the nipple (*M*3, Fig. 27) instead of by suction. The hosecocks at *G*2 (Fig. 23) and *E*3 (Fig. 14) are left open when the buffer is fed in from below through

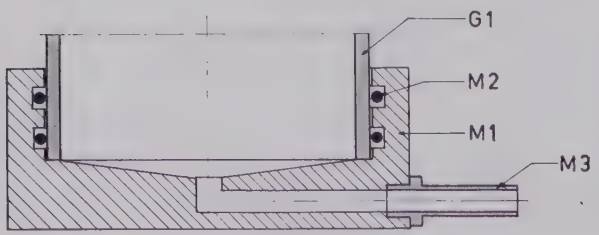


FIG. 27. Bottom piece (*M*) which may be connected to the lower end of the buffer column (*G*, Fig. 10). Its function is to replace the piston (*E*2, Fig. 14).

the nipple (*M*3, Fig. 27). The top of the central cooling tube (*D*7, Fig. 14) may be omitted if a second central cooling tube replaces the piston (*E*2, Fig. 14).

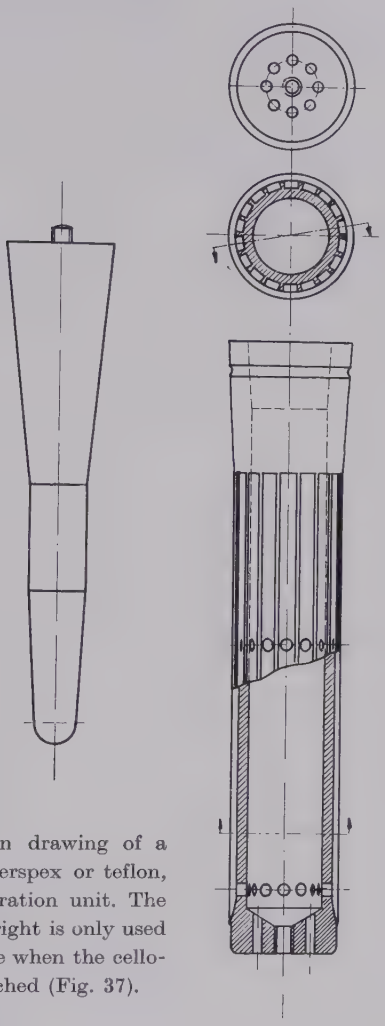


FIG. 28. Construction drawing of a support, made of perspex or teflon, used in the ultrafiltration unit. The conical piece to the right is only used as an auxiliary device when the cellophane tubing is attached (Fig. 37).

Ultrafiltration Apparatus

The apparatus used is a modification of the ultrafiltration unit described by Aronsson (1957). It is made from a solid plexiglass bar, the upper part of which is a smooth-surfaced cone (Fig. 28). Longitudinal furrows are cut, according to the figure, leaving about 1 mm broad rims, and the center is

hollowed out on a lathe. Two or three small openings serve to connect each furrow with the central cavity. The bottom has 6-8 small holes encircling a small central hole which serves to guide a special conical insert. The diameter of the cylindrical support is gauged to fit a standard dialyzing tube.[1] If the support is made of teflon it can be sterilized with the cellophane tubing attached.

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[1] Visking Corporation, Chicago, U.S.A.

METHODS AND MATERIALS

Vertical Preparative Zone Electrophoresis

The only supporting medium used in the author's apparatus was ethanolyzed cellulose[1] (Flodin and Kupke, 1956). This material has good mechanical packing properties and does not contaminate the buffers used with substances which interfere with subsequent determinations of sialic acid and hexosamine content of the different fractions obtained. If the hexose or fucose content of the different fractions is to be studied, polyvinyl chloride[2] may be used as stabilizing medium (Müller-Eberhard and Kunkel, 1956).

The cellulose powder is passed through a 170 mesh standard screen and 500 gram is transferred to a 5 liter, heavy-wall, filtering flask. In a second heavy wall filtering flask 5 liters of the buffer to be used is evacuated with a water suction pump to get rid of dissolved air. The flask with the cellulose powder is evacuated with a vacuum oil pump and the previously evacuated buffer is allowed to fill the evacuated flask containing the cellulose powder. A thin suspension of the cellulose powder in the buffer is thus obtained without any dissolved air bubbles adhering to the particles. The importance of using evacuated buffers has been previously stressed by Flodin and Kupke (1956). The bottom plate with attached thick filter paper (Fig. 16), is evacuated with an oil pump in a desiccator after which the previously evacuated buffer is allowed to fill the bottom of the desiccator. The bottom plate is now immediately screwed on to the separation column with the screws (*B*2, Fig. 13) and the lower end of this column with the attached bottom plate (Fig. 19) is dipped into one of the electrode vessels filled with evacuated buffer. The funnel (*A*1, Figs. 13 and 20) with attached rubber tubing and open hosecock is now screwed to the separation column by means of the threaded collar (*A*2). The hosecock is closed while the lower end of the separation column is still immersed in the electrode vessel filled with buffer. The vessel is then removed. The rubber tubing from the reservoir

[1] J. H. Munktell's cellulose powder, manufactured by Grycksbo pappersbruk, Grycksbo, Sweden.

[2] Geon Resin 426, Goodrich Chemical Corporation, Cleveland, Ohio, U.S.A.

of evacuated buffer is now connected to the rubber tubing at the bottom funnel (*A*1, Figs. 13 and 20) and the electrode vessels may be temporarily removed. Care is taken that no air bubbles are introduced in the system. The evacuated buffer is now allowed to fill the separation column (*C*, Fig. 10) and the inlet piece (*D*) from below. The top piece (*E*, Fig. 10) is removed and replaced by a packing tube (*H*1, Figs. 24 and 29) and the hosecock at the bottom funnel (*A*1, Figs. 13 and 20) is closed. More evacuated buffer fills the packing tube from the nipple (*H*3, Fig. 24) until the bucket (*H*6, Fig. 24) is partly filled with buffer. A thin cellulose suspension is cautiously poured into the bucket where a stirrer rotates slowly. The hosecock at *A*1 (Fig. 13) which has been kept closed is now carefully opened and one or two centimeters of cellulose powder are allowed to sediment on top of the bottom plate. When this is achieved the hosecock at *A*1 is opened fully and a new thin suspension of cellulose in buffer is repeatedly added to the bucket (Fig. 29). When the sedimenting front of cellulose powder (Figs. 12 and 29) is quite close to *C*7 (Fig. 13) the thin cellulose suspension in the packing tube is allowed to drain through the nipple *H*3 (Fig. 24) and the packing tube is filled with fresh evacuated buffer through the same nipple. About 3 or 4 liters of buffer solution are allowed to drain through the separation column. The packing tube (*H*1, Fig. 24) is disconnected and the buffer is allowed to drain to a level one or two millimeters above the upper cellulose level. Three small pieces of filter paper are now placed under the three holes in the lugs of *C*7 (Figs. 13 and 22). This is facilitated by using the long forceps shown in Fig. 20. Through the three holes in *C*7 (Figs. 13 and 22), which are lined with polyethylene tubing, the ends of the needles are adjusted to a level immediately above the cellulose. The central opening at the end of the needles has been closed and instead a small hole has been

Fig. 29. Packing procedure. Note the sedimenting cellulose level.

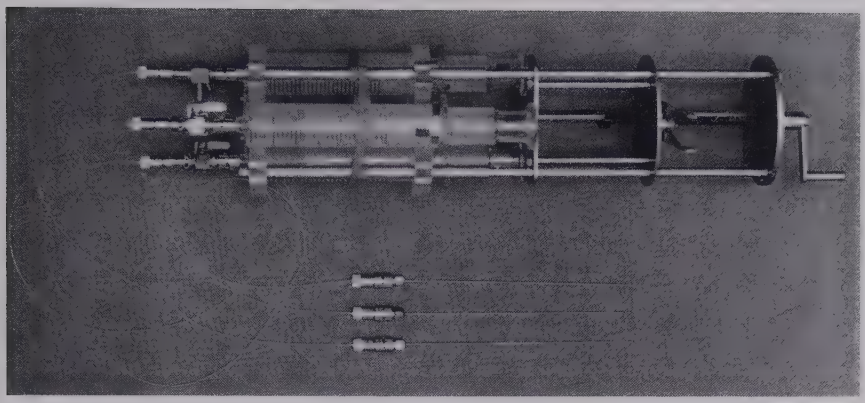

FIG. 30. Three syringes, controlled by a threaded rod, which are used for applying the protein zone to the column.

made in the side wall. This is done to prevent disturbance of the cellulose boundary by an accidental stream of buffer through the needles. The protein zone is applied by means of three 30 ml syringes[1] connected through polyethylene tubings with adapters[2] to the three needles (Fig. 30). The rubber tubing attached at *A*1 (Fig. 20) is connected to a funnel filled with buffer. The buffer surface in this funnel is level with the upper limit of the cellulose powder (Fig. 31). By means of a threaded rod the three syringes deliver the protein solution drop by drop at three symmetrical sites. The level in the funnel (Fig. 31). is adjusted just below the cellulose level, and the protein zone displaces the buffer from the upper section of the separation column filled with cellulose powder. When the protein has thus been applied, buffer is carefully put on top of the protein zone by means of the three syringes.

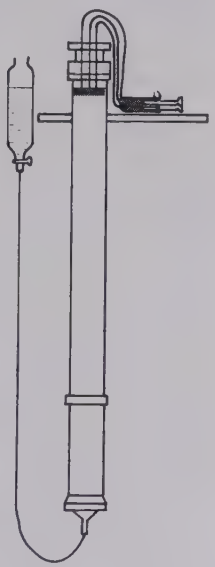

FIG. 31. Schematic drawing of the separation column when the zone is applied from above. The three syringes (Fig. 30) are placed on the guiding bracket (*N*, Fig. 10).

[1] Yale Luer Lok, Becton, Dickinson and Co., Rutherford, N.J., U.S.A.

[2] Clay-Adams, Inc., New York 10, N.Y., U.S.A.

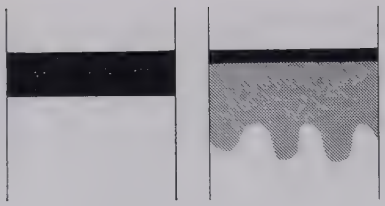

FIG. 32. Gravity influence on the lower zone boundary. Schematic drawing of a coloured protein zone which has been applied to the separation column from above. Initial state to the left. After 16 hours the convectionally unstable lower boundary has formed finger-like protrusions.

It was found that, when 34 ml of serum coloured with bromphenol blue was applied to the separation column from above and left for 16 hours, the upper boundary, as well as a fourth of the width of the original zone, remained perfectly sharp and uniform in colour (Fig. 32). However, the lower boundary of the protein zone which is convectionally unstable, as it rests on a solution with lower density, had been markedly distorted and showed finger-like protrusions around the column. This undesirable spreading may to a certain extent be diminished if the electric field is applied as early as possible, and the zone in the early stage of electrophoresis displaced down the separation column by a slight hydrostatic pressure set up by the buffer pump.

It is advantageous if the electrophoresis is conducted in such a way that the protein components, which are of special interest, are eluted as early as possible. Contaminations with other protein fractions which may have been slightly retarded through reversible adsorption are thus minimized. Losses due to adsorption are also minimized if the component under investigation does not have to pass along the whole length of the column in order to be eluted. Initially sharp, electrophoretically separated zones are always blurred to some extent in the process of elution.

A method for quantitative transfer of a protein zone to the separation column from below is shown in Fig. 33. With this technique it is possible to also stabilize the lower zone boundary through a density shelf made of buffer and an inert substance such as the colloid "Ficoll" (p. 55). Other methods for the application of the zone to the lower end of the column have earlier been described by Coolidge (1939), Haglund and Tiselius (1950) and Porath (1957*b*). Minor components of the protein zone which are irreversible adsorbed by the stabilizing medium may, prior to the electrophoresis experiment, be removed from the sample by means of filtration through

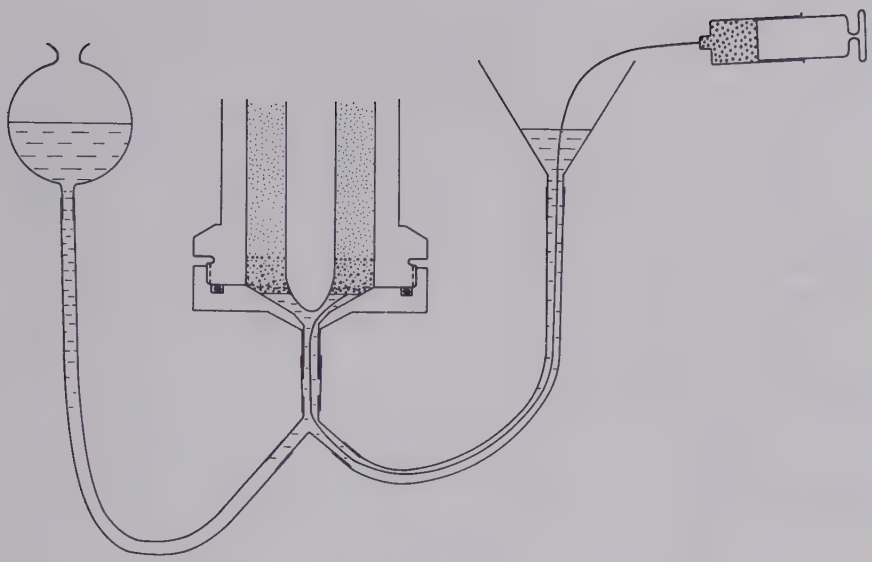

FIG. 33. Schematic drawing of a method for applying a protein zone to the separation column from below. A dense liquid which will not mix with the buffer solution forms a boundary beneath the bottom net of the separation column. The protein zone is applied through thin polyethylene tubing which passes through the dense liquid. The zone may then be displaced by a denser solution made of the buffer and an inert substance such as the colloid "Ficoll" (p. 55).

a small column filled with the stabilizing medium or by use of the extension column described by Porath (1957 *b*).

When the upper boundary of the protein zone has been displaced 1–2 cm, the inlet piece (*D*, Fig. 10) is carefully filled with buffer without disturbing the cellulose powder. The top piece (*E*, Fig. 10) is now attached to the inlet piece (*D*) and the sliding joint (*F*, Fig. 10) makes connection with the buffer column. The buffer column and the horizontal connection have now to be filled with buffer solution. This can be done in two ways. In the old method the piston (*E*2, Fig. 14) is moved down to protect the cellulose level, and the electrode vessel under the buffer column (*G*, Fig. 10) is filled with buffer. The vertical buffer column is filled from below by suction at the nipples *E*3 (Fig. 14) or *G*2 (Fig. 23).

A more advantageous method is to replace the piston (*E*2) with a pyrex cooling tube as described previously (p. 38). The hosecocks at *E*3 (Fig. 14) and *G*2 (Fig. 23) are now left open. An electrode vessel, filled with evacuated buffer, is placed beneath the buffer column (*G*, Fig. 10), and the connecting

piece (Fig. 27) is now attached to the lower end of *G*1 (Fig. 23). The O-rings (*M*2) form a watertight sliding joint. A reservoir of previously evacuated buffer is then connected to the nipple *M*3 (Fig. 27) and buffer is allowed to rise slowly in the vertical buffer column (Fig. 34) until its horizontal connecting part has been filled with buffer. The small forceps shown in Fig. 20 are convenient to make instantaneous closure of the rubber tubing at *E*3. The hosecock at *E*3 (Fig. 14) is then closed. The second electrode vessel is filled with buffer and connected through the pump (Fig. 20) to the first electrode vessel. A second rubber tubing connects the electrode vessels with each other. Constant mixing of the buffer from the anode and the cathode sides is thus obtained when the pump is working (Fig. 35). The levels of the buffer solution in the two electrode vessels are allowed to come into equilibrium. The connecting piece (Fig. 27) is removed from *G*1 and the bottom funnel (*A*1, Fig. 13) is also removed after the hosecock at *A*1 (Fig. 20) has been opened. Both the separation and the buffer column are immersed in their electrode vessels during this operation. The buffer pump is now adjusted to give a slight hydrostatic pressure in the electrode vessel under the buffer column and the electrodes are attached to the power supply. During the first stage of electrophoresis only a little current is allowed to pass through the apparatus and the zone is displaced down the separation column by means of the slight hydrostatic pressure set up by the buffer pump (Fig. 35). During the run, as well as during the packing procedure, cold water at about + 1°C is circulated from a large cooling reservoir with appropriate cooling machinery. The voltage which can be applied to the column is partly limited by the capacity of the cooling machinery. More than one kilowatt has been applied to the present column for several hours without any apparent damaging effect on the separation obtained. About 10 liters of ice had to be added each hour to the cooling bath.

When the separation is completed the fluid content in the separation column is eluted into a fraction collector. The funnel (*A*1, Fig. 13) is screwed on to the separation column. At this stage the lower end of the separation column (*C*, Fig. 10) is still immersed in the buffer of the electrode vessel. The hosecock attached to the nipple *A*1 is kept open when the funnel is screwed on. As soon as a tight seal is obtained between *A*1 and *C*1 (Fig. 13) the hosecock is closed and the electrode vessel is removed to make room for the fraction collector (Fig. 36). It is preferable to have a fraction collector where the tubes can be cooled to + 1°C by the same cooling reservoir which serves to cool the separation column.

Fig. 34.

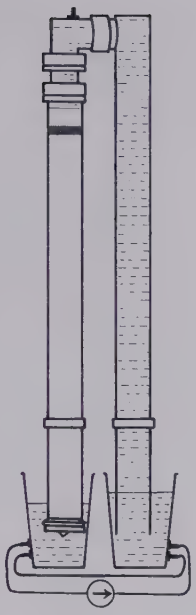

Fig. 35.

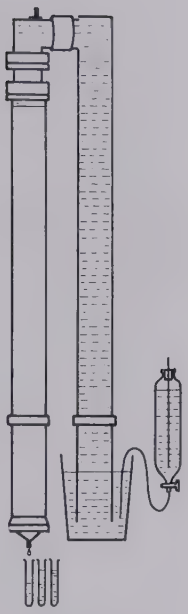

Fig. 36.

Fig. 34. Schematic drawing showing how the bottom piece (*M*1, Fig. 27) is used for filling the buffer column.

Fig. 35. Schematic drawing of the column during a run. The electrode vessels are connected through a pump indicated with an arrow. The bottom funnel of the separation column has been removed. A slight hydrostatic pressure is obtained through the action of the pump and is used to displace the protein zone down the column during the initial stage of the electrophoretic separation. By changing the direction of the pump the electric migration may be suitably balanced.

Fig. 36. Schematic drawing of the elution of the separation column. Three test-tubes indicate a fraction collector.

Ultrafiltration Methods

Unit for 200–300 ml

This type is used for concentration of a solution down to 10–15 ml. A support made of plexiglass or teflon, as described on page 39, is used to support a dialyzing tube.

The dialyzing tube is moistened in hot water at about + 50°C and is pushed over the conical piece and the attached support (Fig. 37). The upper end of the tubing is gently stretched over the conical part and this forms an air-tight seal. The length of the tubing is a little more than twice the length of the support. The conical piece is then removed and the free end of the

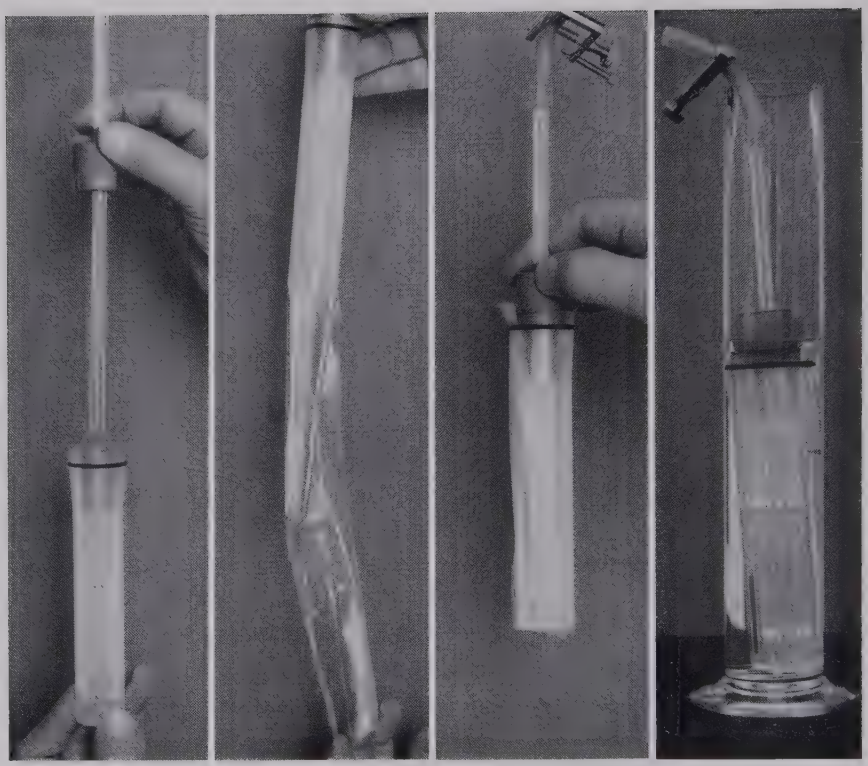

FIG. 37. Ultrafiltration unit for 100–200 ml.

(*a*) A capillary glass tube with a rubber stopper is inserted in the support.

(*b*) The dialyzing tube is pushed over the conical piece and the attached support and is gently stretched over the conical part of the support. The procedure is performed in water at about +50°C.

(*c*) The conical piece has been removed and the free end of the cellophane tubing is fixed in a small furrow at the upper end of the cone of the support with an O-ring gasket.

(*d*) The unit is now placed in a cylinder containing the dilute protein solution and evacuated. It is then left in the cold room. 12 ml of ultrafiltrate is obtained per hour in the central cavity of the support.

dialyzing tube is fixed at the upper end of the cone in a small furrow by means of an O-ring gasket (Fig. 37). A rubber stopper with a central glass capillary which ends at the bottom of the support is inserted. The dilute macromolecular solution is placed in a cylinder together with the ultrafiltration unit (Fig. 37). This unit is now evacuated by means of an oil pump, used also to remove the ultrafiltrate, and when the hosecock is closed the moist dialyzing tube retains the vacuum when left in the cold room.

About 12 ml of ultrafiltrate is obtained per hour in the central cavity of

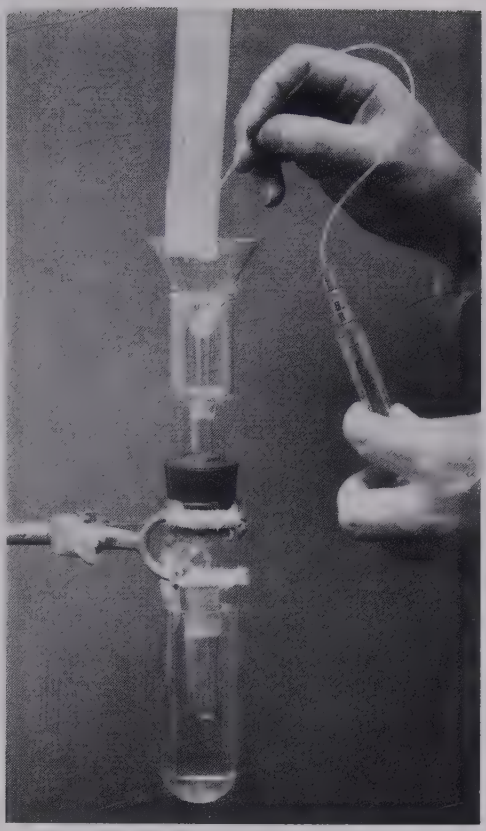

FIG. 38. The procedure used for a quantitative transfer of traces of protein material left in the fold of the cellophane tubing (Fig. 37). The small apparatus in the lower part of the photograph consists of a collodium tube surrounded by buffer solution and is used as a negative pressure dialyzing apparatus (Sörensen, 1917). By means of thin polyethylene tubing attached to a glass syringe the folds of the cellophane tube are rinsed repeatedly with small volumes of buffer solution. The rinsings are concentrated to about 1 ml and the bulk, 15–20 ml, of the previous concentrate obtained in the larger unit (Fig. 37) is then added.

the support. The rubber tubing at the end of the capillary glass tube is connected to an evacuated filtering flask when the central cavity of the support has been filled with ultrafiltrate. The removal of the collected ultrafiltrate is facilitated if the hosecock on the rubber tubing is first carefully opened to allow a few air bubbles to reach the central cavity in the support. The unit has to be evacuated less frequently if the central cavity is connected to a filter flask which is evacuated and left in the cold room at +1°C. An important advantage is that no appreciable dilution takes place in case the cellophane tubing should burst. The dimensions of the above ultrafiltration unit may conveniently be increased to suit larger cellophane tubing.

Unit for 10–20 ml

If further concentration is necessary it is advantageous to use small collodium tubes[1] in a negative pressure dialyzing apparatus (Sörensen

[1] Membranfiltergesellschaft Sartorius-Werke A. G., Göttingen, Germany.

and Höyrup, 1917). A similar type is described by Mies (1953). To get as quantitative a transfer as possible of material adhering to the cellophane membrane the previously described ultrafiltration unit is placed in a clamp over a funnel which is inserted in the open neck of the collodium tube (Fig. 38). The cellophane tubing and especially its folded part at the bottom is rinsed repeatedly with small quantities of buffer (0.2–0.5 ml) by means of a glass syringe with attached polyethylene tubing. This tubing is secured by means of a plastic tubing adapter. The negative pressure dialyzing apparatus is operated at about 300 mm Hg. The rinsings are first concentrated to about 1 ml, after which the bulk of the previous concentrate obtained from the larger unit is added to the Sörensen unit. The collodium tube is surrounded by buffer during the concentration process and this minimizes losses due to dry protein material on the walls of the collodium tube. This little apparatus is especially suited if one wants a high concentration of proteins in a small volume, a requisite for good results in paper and gel electrophoresis.

Determination of Hexosamine

The hexosamine determination is based on the method described by Elson and Morgan (1933), as modified by Blix (1948). Since several chromogens (Kuhn and Krüger, 1957) are formed, all experimental conditions must be rigidly standardized. Slight modifications of the reaction variables may give rise to varying proportions of the involved chromogens. This may be part of the explanation for discrepancies as to the maximum of the absorption peak found in the literature.

Protein fractions obtained by zone electrophoresis are first concentrated by ultrafiltration (p. 48) until a 1–2 per cent solution is obtained. These fractions are then either dialyzed against 0.15 *M* NaCl in the Sörensen apparatus (p. 49) or electrodialyzed with subsequent freeze-drying. The samples are then hydrolyzed in sealed glass ampoules with hydrochloric acid which is added to make the solution 2 *N* in acid.

All samples are taken with the Linderström–Lang constriction pipette (Glick, 1949). The pipettes are calibrated "to contain" or "for delivery". If they are calibrated "for delivery" the absolutely clean and dry pipette is initially filled with the solution to be delivered. This is gently expelled, leaving a more or less viscous film of protein solution on the walls. Mercury is now drawn into the pipette. Some of the liquid which was used to wet the pipette collects on top of the mercury, and the upper end of this liquid is allowed to reach the constriction mark. The mercury is now expelled, thus

leaving the liquid in contact with the walls. The mercury is then weighed and, knowing its density, the volume of the viscous protein solution which may be delivered by the pipette is known (Peters and van Slyke, 1932).

The samples are hydrolyzed for about fourteen hours at 100°C and the hydrolysate is quantitatively transferred to a 5 or 10 ml volumetric flask, depending on the amount of hexosamine present. With serum fractions containing an appreciable amount of hexosamine the determination may be made without the previous chromatographic purification described by Boas (1953). This procedure is extremely valuable in cases where the hexosamine content is very low and interfering substances may dominate the chromogen production (Kent and Whitehouse, 1955). As recommended by Johnson, Ogston and Stanier (1951), the hydrochloric acid may be removed in a vacuum desiccator over sodium hydroxide pellets. Small amounts of distilled water have to be added repeatedly during the procedure to get rid of the hydrochloric acid. The open glass ampoules should have a suitable size and be slightly tilted in the desiccator. If they are thoroughly cooled before being placed in the desiccator the risk of splashing is greatly minimized. This way of removing the hydrochloric acid after hydrolysis has previously been used by the author in studying serum hexosamine levels. It has been found, however, that the method results in reproducible losses of hexosamine of the order of 6 per cent. This may be explained in part by the fact that the hydrochloric acid is being concentrated in the ampoules in the desiccator. Boas (1953) gives details for the stability of the hexosamine in hydrochloric acid of different strengths. The author now uses the neutralization technique described by Boas (1953). In small volumetric flasks the preferably cool samples are cautiously titrated with 4 *N* sodium hydroxide with phenolphthalein as an indicator. A good mixing during the titration is necessary as local excess concentration of sodium hydroxide is extremely dangerous for the alkali labile hexosamine. When the colour turns faintly pink, one or two drops of dilute hydrochloric acid should be added to make the solution colourless. The solution is then made up to the mark with distilled water. A suitable aliquot of this solution, usually one ml, is transferred by means of a constriction pipette to a glass ampoule. One ml of freshly made acetylacetone reagent (Blix, 1948) is added with a constriction pipette and the ampoule is sealed with a flame and acetylated for 45 min. in a large thermostatically controlled water-bath which is vigorously stirred and maintained at $\pm 92°$ (Jorpes, 1935; Boas, 1953).

Five ml of aldehyde-free ethanol, 96 per cent, is added to small test-tubes which are placed in an ice-bath. The acetylated ampoules are chilled in cold

running tap-water, then opened and 1000 μl of the content is taken up with a constriction pipette and delivered into cold ethanol. Thorough mixing is thus achieved. 500 μl of cold Ehrlich's reagent, which has been kept in the ice-bath, is now added to and mixed with the content of the tubes. The colour may then be further developed either at +60°C for 5–10 min. (Rondle and Morgan, 1955), or at +37°C for an hour (Boas, 1953). The same absorbance value at 535 mμ will be obtained but a slightly altered shape in the absorption curve has been noted, indicating that the ratio between the different chromogens may not be identical. Two isobestic points were obtained at 445 and 585 mμ respectively. This phenomenon perhaps depends on the type of heat-treatment employed. However the method is extremely reproducible and the ordinary error encountered, if calibrated constriction pipettes are used, is dependent on the photometric error inherent in all colorimetric methods.

Determination of Sialic Acid

Sialic acid is determined by means of a modified Bial reaction (Werner and Odin, 1952), which has been slightly modified by Laurell, Laurell, and Waldenström (1957), to suit the Linderström–Lang constriction pipettes.

Protein fractions obtained by zone electrophoresis are dialyzed against 0.15 *M* NaCl, followed by ultrafiltration in the unit described on p. 49. Four ml of a suitable concentration of the solution is used directly with one ml of the Bial reagent. Four ml of a suitable concentration of the sample in 0.15 *M* NaCl is then added to a ten ml glass-stoppered tube and mixed with one ml of the Bial reagent. The tubes are submerged for 10 min. in a large thermostatically controlled bath which is vigorously stirred and maintained at +108°C. Teflon sleeves on the glass stoppers greatly facilitate the opening after the reaction has been completed. To prevent bumping, the tubes, which are all of the same size, are placed on a rack covered with a layer of thick synthetic sponge rubber with a heavy metal lid on top. The coloured material is extracted in purified amyl alcohol as described by Werner and Odin (1952). To prevent slight cloudiness which may interfere with colorimetric determinations, aliquots of dry amyl alcohol may be added to each of the tubes as recommended for copper determinations by C. B. Laurell. The method gives reproducible results with the same sample. With pure sialic acid, linear calibration curves are obtained, but if dilutions of serum protein fractions containing sialic acid are made in 0.15 *M* NaCl, a straight-line relationship is not obtained for absorbance values higher than 0.35. Already with absorbance values of 0.3 there is an

appreciable deviation from the linear relationship. Variations in the temperature of the bath, the time of heating, and the concentrations of the different reagents have all been investigated. Although even a higher specific extinction may be obtained by such adjustments this does not alter the fact, that there is no linear relationship between concentration and absorbance values above 0.3.

Other Determinations

Hexoses

Hexoses are semiquantitatively determined in the presence of protein by the method described by von Holt (1954).

Haptoglobin

Haptoglobin has been determined by Jayle's method (1951). The author, during a visit at the Malmö clinic, is indebted to Dr. M. Nyman for help and advice in connection with the haptoglobin determinations.

Other Methods

Starch Gel Electrophoresis

Zone electrophoresis in starch gel has been performed according to Smithies (1955). The author is indebted to Dr. C. B. Laurell for technical details in connection with starch gel electrophoresis and for the first determinations which were made in the Malmö clinic with a batch of starch prepared in that laboratory. A suitable starch has recently been obtained commercially.[1]

Paper Electrophoresis

Paper electrophoresis has been performed according to conventional methods (Laurell, 1957). Paper electrophoretic strips stained for "carbohydrates" have been eluted and evaluated according to Laurell and Skoog (1956).

Sedimentation Experiments

Sedimentation experiments have been performed in a Spinco ultracentrifuge. When the solution leaving the zone electrophoresis column had a concentration of 0.5–1 per cent, the samples were run as they left the column. In other cases they have been concentrated by ultrafiltration (p. 48).

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[1] Starch-hydrolysed, Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

Moving Boundary Electrophoresis

The moving boundary experiments reported in Figs. 1 and 2 have been performed in an electrophoresis apparatus built at the Institute. Figs. 50 and 51 have been obtained with an apparatus manufactured by LKB, Stockholm.

Buffer Solutions

Acetate buffer, pH 4.0, ionic strength $\mu = 0.1$

Glacial acetic acid	25.99 ml
Sodium acetate, anhydrous	13.61 g

Acetate-EDTA buffer, pH 4.5, ionic strength $\mu = 0.1$

Acetic acid 1 *M*	125 ml
Sodium acetate 1 *M*	100 ml
EDTA	1.0 g

Barbiturate buffer, pH 8.6, ionic strength $\mu = 0.1$

Sodium barbiturate 0.2 *M*	250 ml
HCl 0.2 *M*	30 ml

Barbiturate-Ca buffer, pH 8.6 (Laurell, Laurell, and Skoog, 1956)

Sodium diethylbarbiturate	8.76 g
Diethylbarbiturate	1.38 g
Calcium lactate	0.384 g

Citrate-phosphate buffer, pH 4.5

Citric acid $H_3C_6H_5O_7 \cdot H_2O$	7.82 g
$Na_2HPO_4 \cdot 2H_2O$	10.5 g

Citrate-phosphate-EDTA buffer, pH 4.4

Citric acid $H_3C_6H_5O_7 \cdot H_2O$	7.82 g
$Na_2HPO_4 \cdot 2H_2O$	10.5 g
EDTA	1.0 g

Tris-EDTA buffer, pH 9.0 (Aronsson and Grönwall 1957)

tris(hydroxymethyl)-aminomethane $(CH_2OH)_3CNH_2$	60.5 g
EDTA	6.0 g
H_3BO_3	4.6 g

In each case the quantities given were dissolved in sufficient distilled water to make one liter of solution.

Sera

Sera used in the experiments reported in Figs. 45 and 46 were immediately dialyzed against the buffer used in the following electrophoresis experiments. A slight precipitate formed during dialysis.

All other sera were frozen and stored at -20 °C prior to use.

EXPERIMENTAL RESULTS

As described in the previous section the zone can be applied to the column either from below or from above, but in the separations reported here the zone has been applied from above. The method for the application of the zone from below was not developed at the time when the runs were made. In general, the application of the zone from below has many advantages because it is then easier to get a well defined zone as both the upper and lower boundaries of the protein zone may be stabilized through density gradients. A suitable concentration of the inert colloid, "Ficoll",[1] in buffer gives a slightly higher density than has the dialyzed protein solution and thus the lower boundary of the protein zone is stabilized.

Filtration Experiment

A zone consisting of 45 ml of serum, which had been previously dialyzed against citrate phosphate buffer (p. 54) at pH 4.5, was applied to the vertical column from above by means of the three syringes (Fig. 30). The vertical separation column was then filled with ethanolyzed cellulose powder suspended in the same citrate-phosphate buffer. The length of the column containing the stabilizing medium is 140 cm and the buffer volume in this region is approximately 3500 ml. The protein zone was slowly displaced and eluted from the column at the rate of 30–40 ml per hour. In this case the lower boundary of the protein zone was partly stabilized by a small zone of 30 ml of the same buffer containing 4 per cent (w/v) sucrose. Concentrations in the different fractions were measured through absorbance values at 280 mμ (Fig. 39). This sucrose buffer solution had a slightly higher density than had the protein solution. Immediately after the boundary between the sucrose solution and the protein zone was formed it was slowly compensated down the column in order to minimize "the droplet effect", first observed by Svensson, Hagdahl, and Lerner (1957) in density gradient columns. Their explanation was the following. Sucrose diffuses into the protein zone with greater velocity than the protein diffuses into the sucrose and this causes "the droplet effect". As mentioned above, the

[1] Obtained from Pharmacia, Copenhagen-Vanlöse, Denmark.

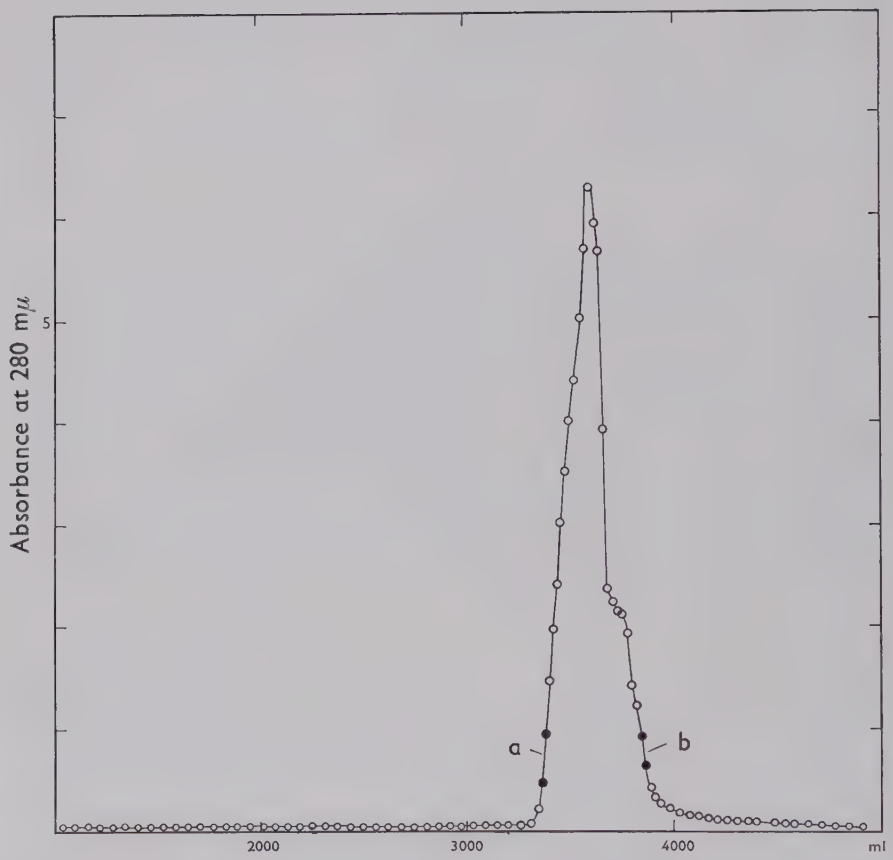

Fig. 39. Filtration experiment. 45 ml of serum was applied to the column from above and eluted at the rate of 30–40 ml per hour into a fraction collector. The lower boundary was partly stabilized by a zone of 30 ml of buffer containing 4 per cent (w/v) sucrose. Citrate-phosphate buffer (p. 54) at pH 4.5 was used for the elution.

inert high molecular weight colloid "Ficoll" may give better results than sucrose if used for this purpose.

The asymmetrical pattern obtained (Fig. 39) shows clearly that a certain amount of adsorption had taken place in the column. Tubes from the front of the eluted zone (*a*) and from the rear end (*b*) were concentrated by ultrafiltration. Paper electrophoresis patterns of these fractions are shown in Fig. 40. As seen from the above patterns, the front of this zone (*a*) contains appreciably more albumin than the fraction obtained from the rear portion (*b*). The peak in Fig. 39 showed the usual bilirubin colour for albumin, followed by a slightly reddish tint, which indicates the presence of transfer-

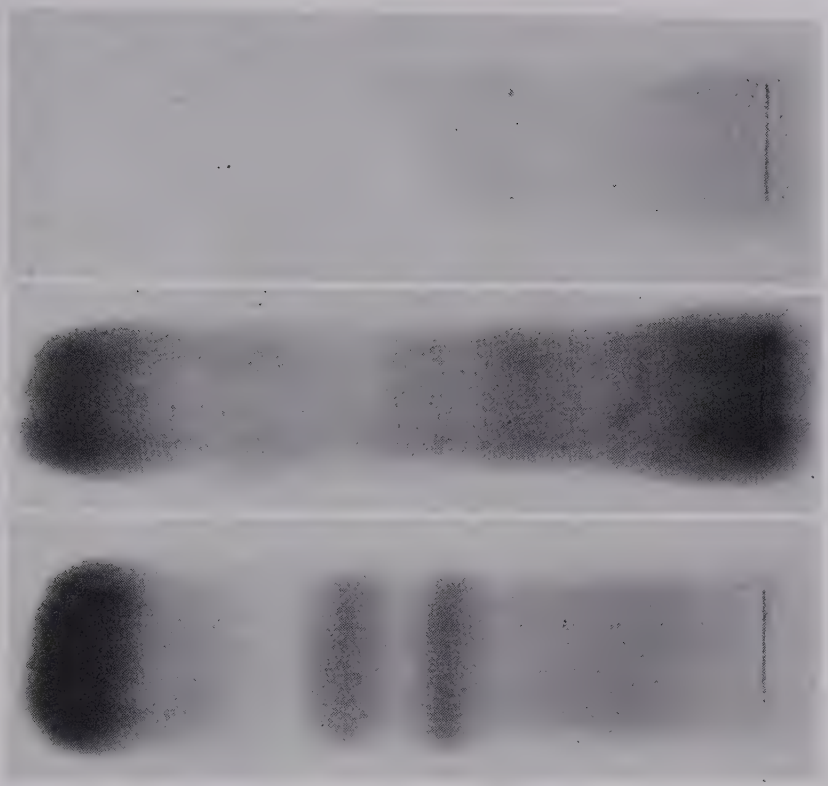

FIG. 40. Paper electrophoretic patterns in barbiturate-Ca buffer (p. 54) at pH 8.6, ionic strength $\mu = 0.05$.

A. The fraction at *a*, Fig. 39, after concentration in the small ultrafiltration unit (p. 49). The starting position is indicated by a pencil line.

B. The fraction at *b*, Fig. 39, was concentrated in the same way. The upper part of the figure shows this fraction run at two different concentrations. It illustrates the fact that in paper electrophoresis, because of adsorption to the paper, it is important to apply a concentrated solution to get good patterns.

rin. The broad shoulder to the right in Fig. 39 shows that some of the material apparently lags considerably behind because of chromatographic effects. It should be pointed out here that some of the above chromatographic effects of the ethanolyzed cellulose powder may be due to the small sucrose buffer zone, which was used to stabilize the lower boundary of the serum.

Zone electrophoresis of sera in citrate-phosphate buffer at pH 4.5

(*a*) Fig. 41 shows the pattern obtained with 36 ml of serum from a patient with rheumatoid arthritis in citrate-phosphate buffer (p. 54) at pH 4.5. The elution rate was 30 ml per 20 min.

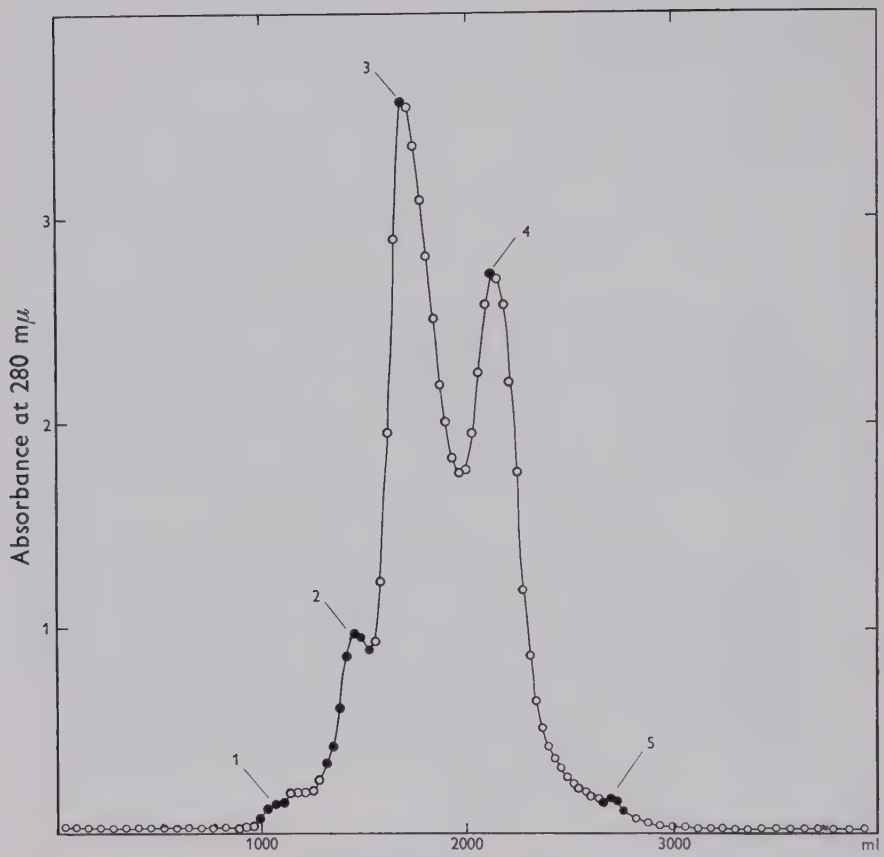

FIG. 41. Zone electrophoresis of serum in citrate-phosphate buffer (p. 54) at pH 4.5. 36 ml of serum from a patient with severe rheumatoid arthritis was applied to the column from above. The experiment was run for 43 hours and the total energy passed was 16.2 kilowatt hours.

Two peaks (1 and 2, Fig. 41) migrate in front of the albumin fraction (3). The sedimentation pattern obtained with material from peak 1 is shown in Fig. 49*C*. The sedimentation constant $S = 2.8$ for a 0.8 per cent solution in citrate-phosphate-EDTA buffer (p. 54) at pH 4.4. The sedimentation constant $S = 3.0$ for a 0.75 per cent solution in the same buffer. Material from peak 2 was found to move as alpha-2 globulin in paper electrophoresis. The sedimentation patterns obtained with a 0.9 per cent solution of fraction 2 showed two components with sedimentation constants $S = 2.2$ and $S = 3.9$ respectively when examined in tris-EDTA buffer (p. 54). Fraction 3 consists of albumin and a small amount of matèrial with sedimentation constant $S = 18$ in the same buffer.

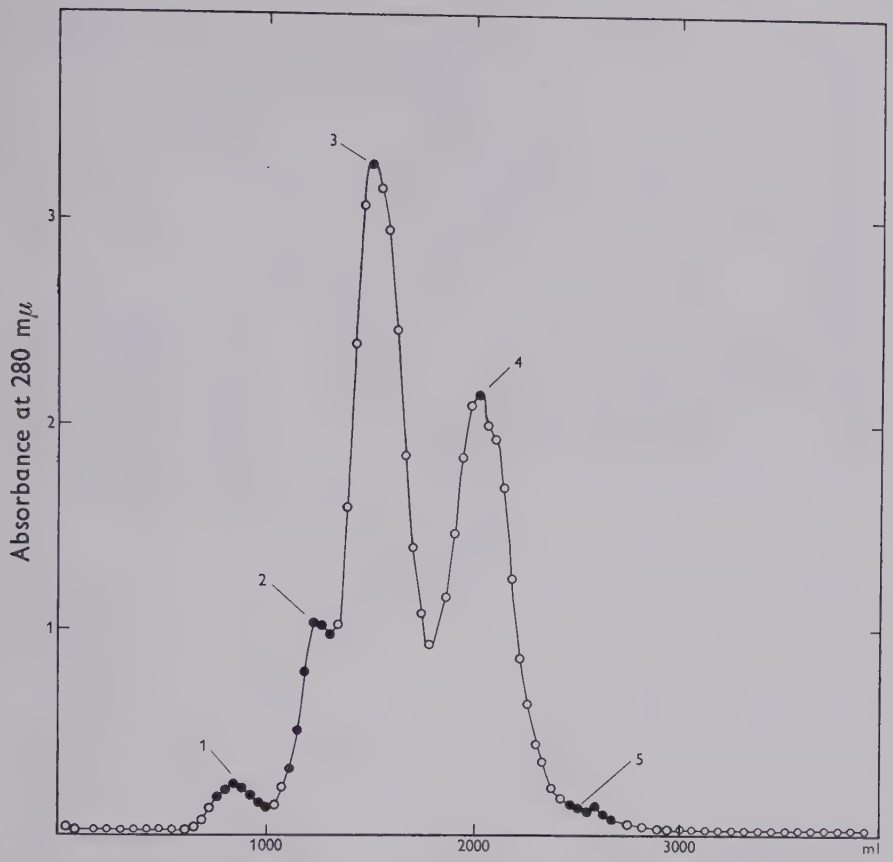

FIG. 42. Zone electrophoresis of serum in citrate-phosphate buffer (p. 54) at pH 4.5. 37 ml of serum from a patient with severe rheumatoid arthritis was applied to the column from above; 160 hours, 21 kWh. An ultracentrifugal pattern of fraction 1 is shown in Fig. 47*A*. Ultracentrifugal patterns of fraction 2 are shown in Figs. 47*C* and *D*. This fraction moved in paper electrophoresis in barbiturate buffer as an alpha-2 globulin, Fig. 44. It was shown in starch gel electrophoresis, according to Smithies (1955), to have bands typical for haptoglobin 2-2. Fractions 3, 4 and 5 as in Fig. 41.

(*b*) Fig. 42 shows the pattern obtained with 37 ml of serum from a patient with rheumatoid arthritis in a citrate-phosphate buffer at pH 4.5 (p. 54). The elution rate was 30 ml per 20 min.

Two peaks (1 and 2, Fig. 42) shown to the left migrate in front of the albumin peak (3). The sedimentation pattern obtained with material from peak 1 is shown in Fig. 47*C*. The sedimentation constant $S = 2.6$ for a 1 per cent solution in citrate-phosphate-EDTA buffer (p. 54), while the

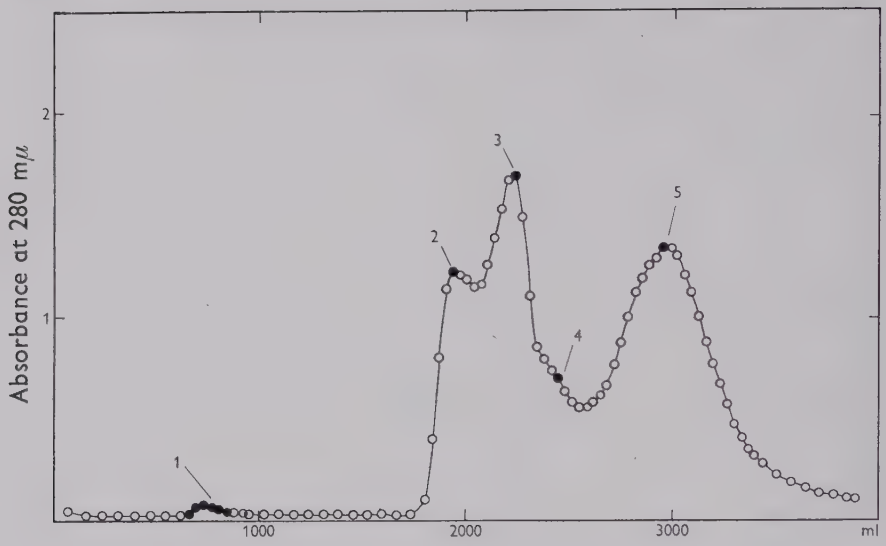

Fig. 43. Zone electrophoresis of serum in acetate-EDTA buffer (p. 54) at pH 4.5.

34 ml of serum from the same patient as in Fig. 42 was applied to the column from above. The albumin was in this case very slightly stained with bromphenol blue; 134 hours, 29 kWh. Fraction 1 consists of orosomucoid. Its ultracentrifugal pattern is shown in Fig. 47 *D*. The paper electrophoretic pattern obtained with fraction 2 in barbiturate buffer at pH 8.6 is shown in Fig. 44. This fraction consists mainly of albumin with traces of alpha-2 globulin. The alpha-2 globulin was shown in gel electrophoresis to be the slow alpha-2 component (Smithies, 1955). Ultracentrifugal patterns obtained with fractions 2 and 3 are shown in Figs. 48 *E* and *F*. Both fractions contained 1–2 per cent of material with a sedimentation constant of about $S = 19$. Fractions 2 and 3 in cellulose strips (Kohn, 1957) with tris-EDTA buffer (p. 54) were shown to consist almost exclusively of albumin.

sedimentation constant for a 0.9 per cent solution in acetate-EDTA buffer (p. 54) is $S = 2.6$.

Material from peak 2 was found to move as alpha-2 globulin in paper electrophoresis in barbiturate-Ca buffer at pH 8.6 (p. 54). Theelectrophoretic pattern for such a sample is shown in Fig. 44 *B*. In starch gel electrophoresis (Smithies, 1955) this fraction showed all the bands characteristic for haptoglobin 2–2. The sedimentation pattern of this fraction contained at least four components.

A 1 per cent solution of the material in peak 3 in citrate-phosphate buffer (p. 54) showed a main component corresponding to albumin with a sedimentation constant $S = 4.0$ and 1–2 per cent of rapidly sedimenting material with sedimentation constant $S = 17$.

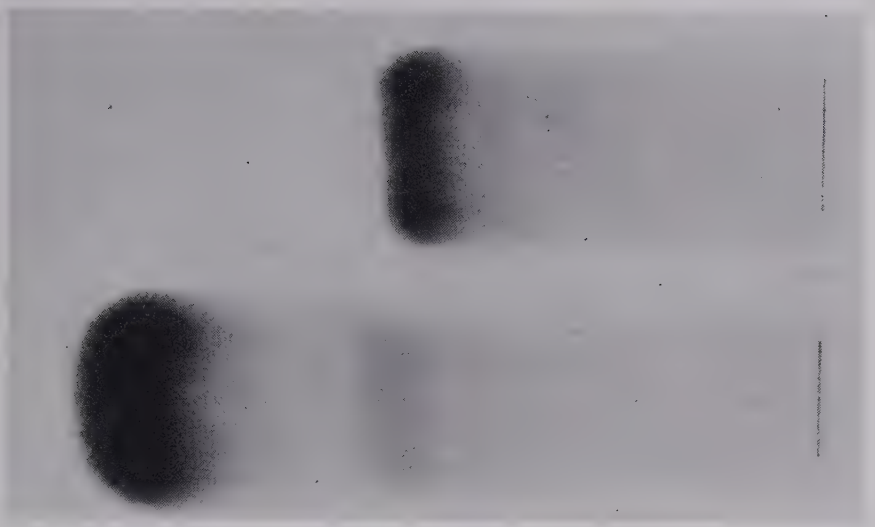

FIG. 44. Paper electrophoretic patterns obtained in barbiturate-Ca buffer (p. 54) at pH 8.6, with fraction 2, Fig. 42 (above) and fraction 2, Fig. 43 (below). It should be noted that the fractions have been obtained from the same serum in different buffers at pH 4.5.

Peak 4 shows a main component with sedimentation constant $S = 4.6$ when a 1 per cent solution in tris-EDTA buffer was used. Two minor components with $S = 3.3$ and 15 respectively were also obtained in this fraction.

The slow moving peak 5 shown in Fig. 42 may be compared with the corresponding peak obtained from the experiment reported in Fig. 41. This peak was dialyzed against tris-EDTA buffer (p. 54) and concentrated by ultrafiltration. The sedimentation pattern of a 0.7 per cent solution of component 5 in tris-EDTA buffer (p. 54) showed a main peak with a sedimentation constant $S = 4.9$ and two minor peaks with $S = 3.4$ and 14, respectively. The sedimentation pattern obtained with corresponding material isolated from the rear end of the gamma globulin fraction in Fig. 46 only showed two components with $S = 4.6$ and $S = 13$ when a 0.7 per cent solution in tris-EDTA buffer (p. 54) was examined.

Zone electrophoresis of sera in acetate-EDTA buffer at pH 4.5

(*a*) An experiment with serum from the same patient as in Fig. 42 is shown in Fig. 43, but in this case acetate buffer with EDTA (p. 54) was used. The peak 1 in the diagram consists of orosomucoid, M1 according to Winzler's nomenclature, and its sedimentation constant was found to

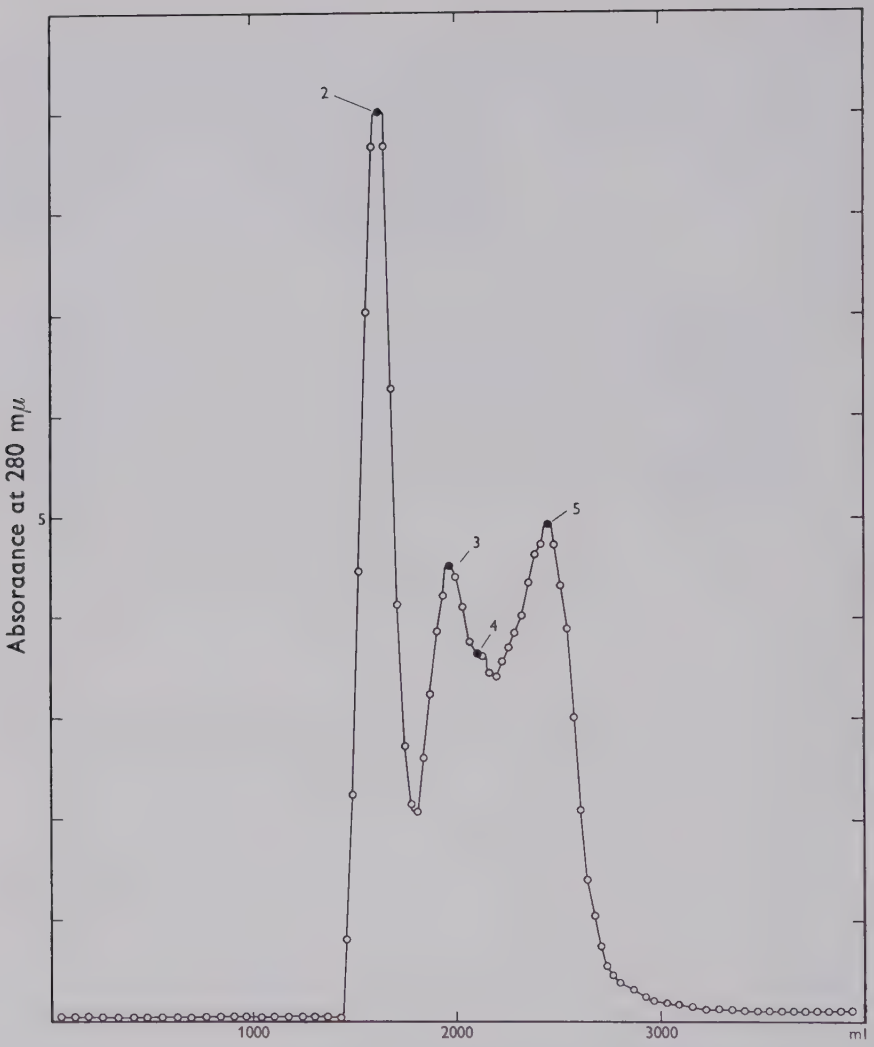

FIG. 45. Zone electrophoresis of serum in acetate-EDTA buffer (p. 54) at pH 4.5.

100 ml of serum from an apparently healthy person was applied to the column from above. 132 hours, 14 kWh. The moving boundary pattern obtained in acetate buffer at pH 4.5, ionic strength $\mu = 0.1$, is shown in Fig. 2 *B*. Ultracentrifugal patterns of fractions 2 and 3 are shown in Figs. 48 *C*, *D* and *A*, *B*, respectively. These patterns show a great similarity to the corresponding patterns obtained from fractions 2 and 3 in Fig. 43. Note the varying ratio between components 2 and 3 in Fig. 43 and Fig. 45. Cellulose acetate strips (Kohn, 1957), obtained with fractions 2 and 3 in tris-EDTA buffer showed that fraction 3 contained albumin and beta globulin and that fraction 2 contained albumin, alpha-2, and beta components. It should be noted that no M 1 fraction could be found even though three times as much serum was applied as in the experiment reported in Fig. 43.

be $S = 3.0$, Fig. 47 *D*. The peak 2 was found to consist of albumin and alpha-2 globulin when examined by paper electrophoresis. In starch gel electrophoresis this alpha-2 globulin moved as the slow alpha-2 globulin (Sα–2). The sedimentation patterns of peaks 2 and 3 are shown in Fig. 48, *E* and *F*. The paper electrophoretic pattern is shown in Fig. 44 *A*. The pattern obtained in Fig. 43 should be compared with the one in Fig. 45 since the same buffer was used in both experiments. The difference in orosomucoid content is readily observed and the discrepancies in the albumin pattern in the two cases should be noted. A 0.85 per cent solution of the material in peak 5 in Fig. 43 was obtained in tris-EDTA buffer (p. 54) and its sedimentation constant was found to be $S = 4.7$.

(*b*) 100 ml of serum from an apparently healthy person were applied to the column from above. The experiment was run for 132 hours and the total energy passed was 14 kWh. The elution rate was 30 ml per 20 min. The pattern obtained is shown in Fig. 45. The ultracentrifugal patterns of fractions 2 and 3 are shown in Figs. 48 *C*, *D* and *A*, *B*, respectively. Cellulose acetate strips (Kohn, 1957) obtained with fractions 2 and 3 in tris-EDTA buffer showed that fraction 2 (Fig. 45) contained mainly albumin but also some alpha-2 and beta globulins and that fraction 3 also contained mainly albumin, a small amount of alpha-2, and considerably more beta globulin than fraction 2. The M1 fraction could not be detected even though three times as much serum was applied to the column as in the experiment reported in Fig. 43.

Zone electrophoresis of serum in citrate-phosphate-EDTA buffer initially at pH 4.4 and gradually adjusted to pH 6.5.

95 ml of serum from the same person as in Fig. 45 were applied to the column from above. The experiment was run for 208 hours and the total energy passed was 25 kWh. By means of the buffer pump the zone was slowly displaced down the column. During the last 24 hours of the experiment the pH was slowly adjusted to 6.5. This was possible since the citrate ion slowly decomposed at the electrode under the influence of the electric current. The buffer capacity changed from 0.039 to 0.016. The elution rate was 30 ml per 20 min. The pattern obtained is shown in Fig. 46. From the region between tubes nos. 7 and 17, 3.4 gram of albumin was obtained. Ultracentrifugal patterns from tubes nos. 7, 11, and 17 are shown in Figs. 49 *D*, *A* and *B*, respectively. No traces of material with a sedimentation constant higher than that of albumin could be observed, and the boundaries were found to be symmetrical even when enlarged. Moving boundary patterns of the fraction from tube no. 11 are shown in Figs. 50 and 51.

DISCUSSION

The described zone electrophoresis apparatus may be used for preliminary separations of serum samples of the order 20–200 ml.

Only under especially favourable buffer conditions may homogeneous, well-defined serum components be obtained in such a separation as has been reported here for the orosomucoid, the haptoglobin, and the albumin fractions. However, a complete separation may often be obtained by means of a second fractionation in zone electrophoresis at another pH or at the same pH but in another ionic environment.

Recent developments in the field of protein chromatography have amply demonstrated the applicability of this technique to certain procedures used for the isolation of well-defined components from individual sera. The technique is particularly useful in combination with a preliminary electrophoretic separation (Tiselius, 1958). If, however, large quantities of individual sera are available, a fractionation by means of precipitation procedures may be recommended if these procedures have been shown not to denature the fraction of interest. Modern developments in the field of plasmaphoresis (Tullis *et al.*, 1956) have made this approach feasible for human sera. The isolation even of minor serum components which are present in concentrations as low as 100 microgram per milliliter may thus be achieved. Genetically controlled differences in molecular structure such as have recently been described for haptoglobin by Smithies (1956) and for albumin by Knedel (1958) may be studied in detail.

Goodman, Ramsey, and Poulik (1958) have presented most interesting data suggesting antigenic microheterogeneity of albumin, orosomucoid, ceruloplasmin, transferrin and several gamma globulins isolated from pooled sera. Some of the microheterogeneities reported in the literature may depend on storage effects with partial denaturation of some proteins or bacterial contamination with *B. subtilis* giving rise to bacterial proteolytic enzymes. However, real genetically controlled minor modifications of at least some of these proteins will most probably be found. Smithies (1957) has observed considerable racial differences in the frequency of occurrence in the serum of beta-globulin D. The observed differences have now proved to be genetically controlled and Horsfall and Smithies (1958) and Smithies

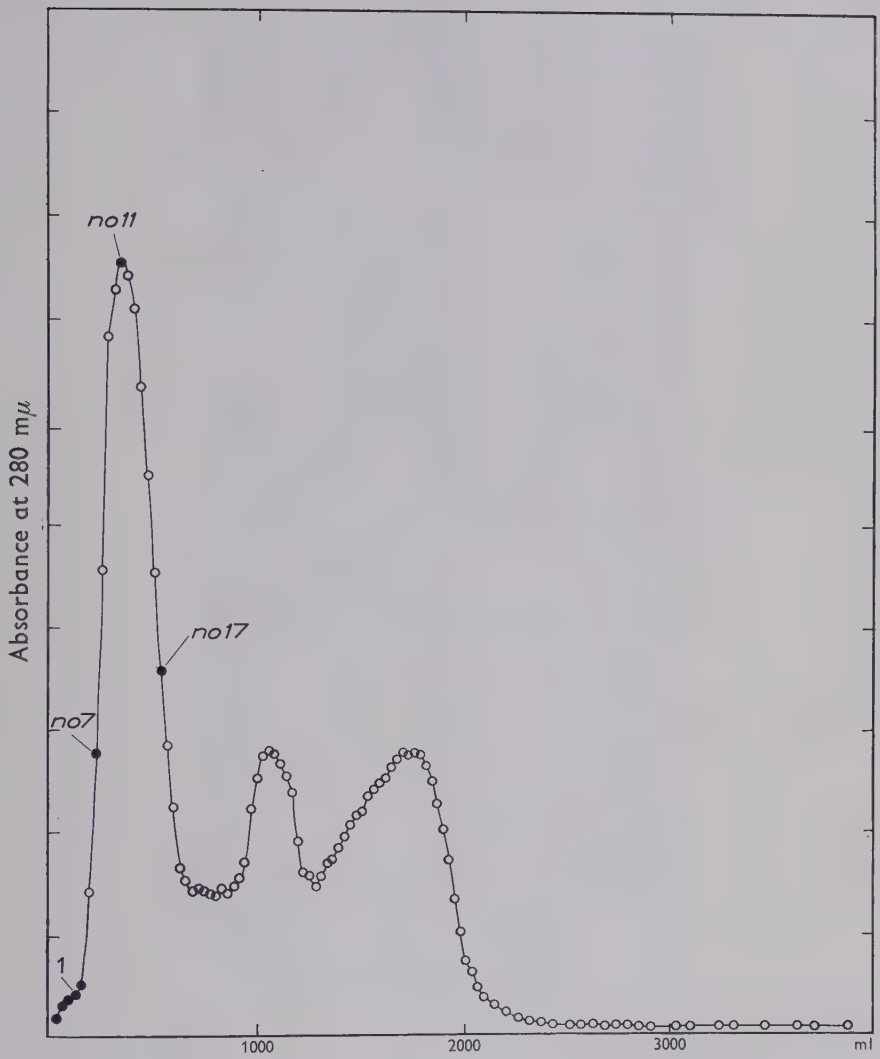

FIG. 46. Zone electrophoresis of serum in citrate-phosphate-EDTA buffer (p. 54) at initial pH 4.4.

95 ml of serum from the same healthy person as in Fig. 45 was applied to the column from above; 208 hours, 25 kWh. During the last 24 hours of the run the pH was slowly adjusted to 6.5. This was possible since the citrate ion slowly decomposed at the electrode under the influence of the electric current; at the same time the buffer capacity was but slightly changed. 3.4 gram of albumin was obtained in the region between tubes nos. 7 and 17. Ultracentrifugal patterns from tubes nos. 7, 11, and 17 are shown in Figs. 49 *D*, *A*, and *B*, respectively. No traces of material with a higher sedimentation constant could be observed, and the boundaries were found to be symmetrical even at closer inspection. Moving boundary patterns of the fraction from tube no. 11 are shown in Figs. 50 and 51.

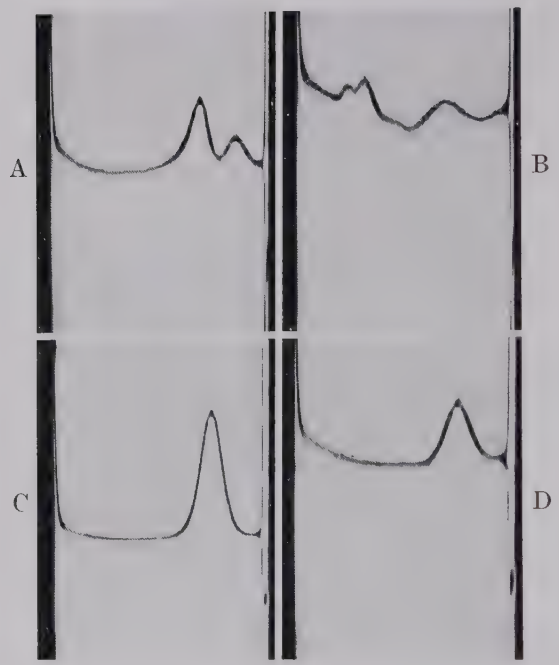

FIG. 47. Sedimentation patterns obtained with fractions shown in Figs. 42 and 43 at 59,000 r.p.m. Protein concentration about 0.8 per cent (w/v).

	Fraction	Buffer (p. 54.)	pH	Min.	θ	S_{20}
A.	2, Fig. 42	citrate-phosphate	4.5	37	40°	2.9
B.	2, Fig. 42	citrate-phosphate	4.5	85	30°	6.8 7.7
C.	1, Fig. 42	acetate-EDTA	4.5	86	50°	2.6
D.	1, Fig. 43	acetate-EDTA	4.5	75	30°	2.9

(1958) suggest that there is a locus for beta-globulins at which any of three alleles may be present (β^B, β^C, or β^D).

The method for labelling organic molecules with tritium gas (Wilzbach, 1957; Bergström and Lindstedt, 1957) which has also been used for "the T labelling" of ribonuclease, may be useful for labelling homogeneous serum components from individual sera. Zone electrophoresis at the isoelectric point of a protein so labelled may have certain advantages compared to chromatography for the isolation of the required labelled molecules since exchangeable tritium may be removed at the same time as the isolation is obtained.

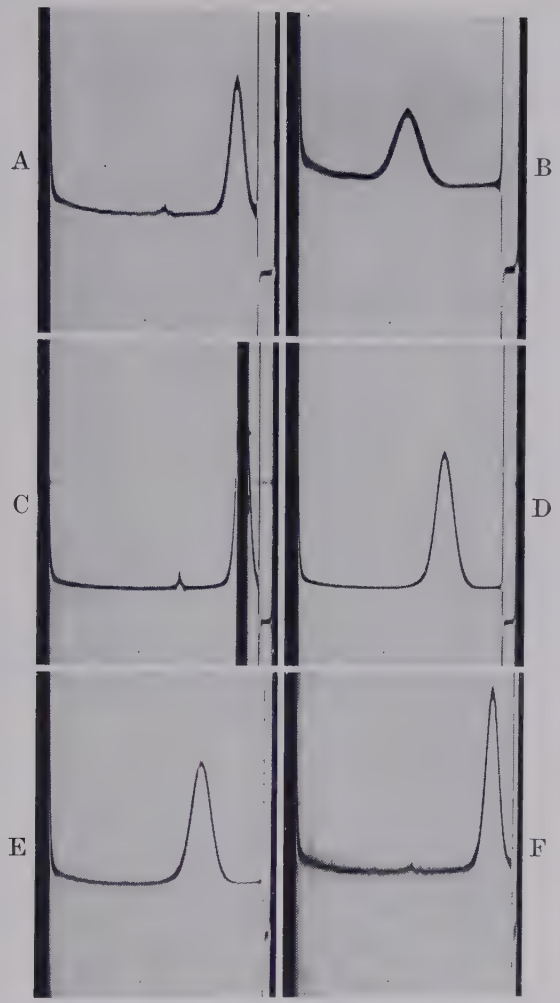

FIG. 48. Sedimentation patterns obtained with fractions shown in Figs. 43 and 45 in acetate-EDTA buffer (pH 4.5) at 59,000 r.p.m. Protein concentration about 0.8 per cent (w/v).

	Fraction	Min.	θ	S_{20}
A.	3, Fig. 45	24	50°	3.8
B.	3, Fig. 45	104	40°	18
C.	2, Fig. 45	18	65°	3.9
D.	2, Fig. 45	66	65°	18
E.	2, Fig. 43	69	55°	4.0
F.	3, Fig. 43	22	50°	18

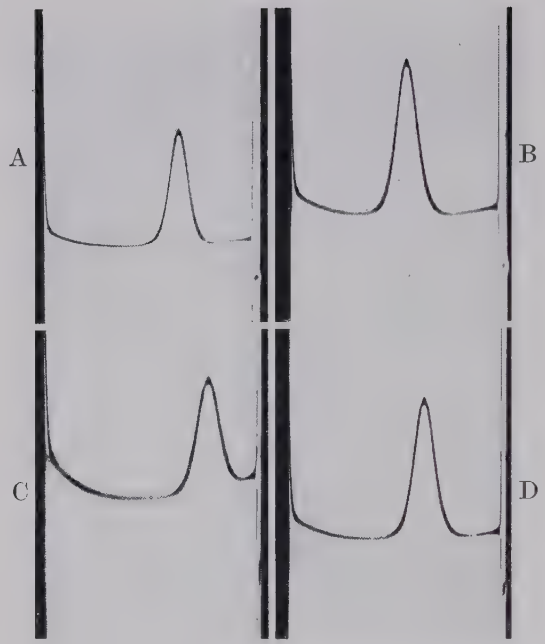

FIG. 49. Sedimentation patterns obtained with fractions shown in Figs. 41 and 46 at 59,000 r.p.m. Protein concentration about 0.8 per cent (w/v)

	Fraction	Buffer (p. 54.)	pH	Min.	θ	S_{20}
A.	Tube no. 11, Fig. 46	citrate-phosphate-EDTA	6.5	87	65°	3.5
B.	Tube no. 17, Fig. 46	citrate-phosphate-EDTA	4.4	103	55°	3.3
C.	1, Fig. 41	acetate-EDTA	4.5	86	40°	2.8
D.	Tube no. 7, Fig. 46	citrate-phosphate-EDTA	4.4	82	55°	3.8

If the protein zone is not observed during the electrophoretic separation a preliminary experiment is usually performed in order to discover the approximate rate of migration which, of course, is also affected by the electro-osmotic flow. This latter effect may be visualized as described by Flodin and Kupke (1956). The rate of migration of serum albumin may often be directly observed due to its content of bilirubin. Bromphenol blue or certain porphyrins (Bennhold, 1938) added to the serum stain the migrating albumin zone.

The method of Agner (1948) for semi-quantitative estimation of protein based on the protein error of indicators has recently been used for detection of protein in urine (Free and Fonner, 1958). The same principle may be

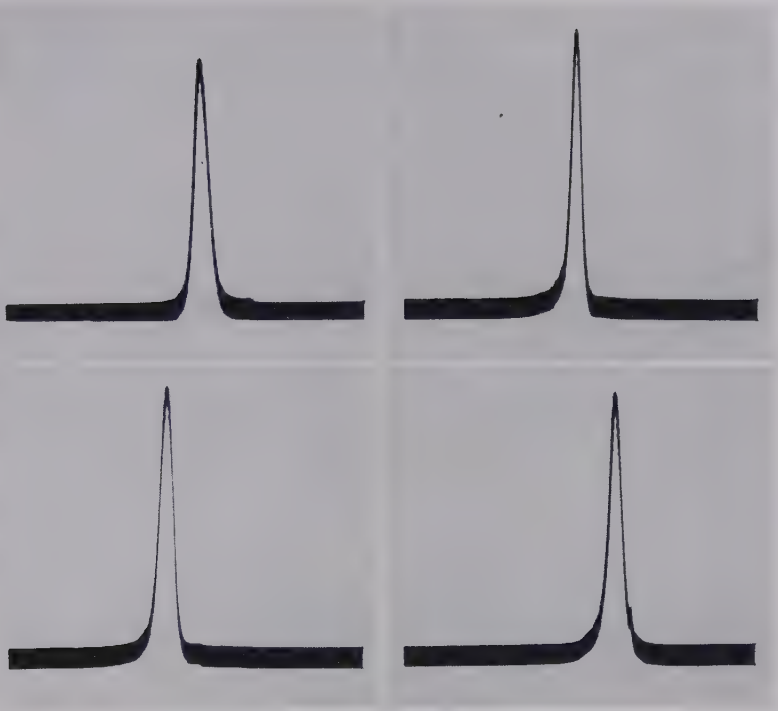

FIG. 50. Moving boundary patterns obtained with the albumin fraction from tube no. 11 in Fig. 46.

A. Pattern obtained in acetate buffer at pH 4.5, ionic strength $\mu = 0.1$; 103 min. at 20 mA. Mobility $= 0.35 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}. Ascending side to the right.

B. Pattern obtained in citrate-phosphate-EDTA buffer (p. 54) at pH 4.4; 155 min. at 20 mA. Mobility $= 0.5 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}. Ascending side to the right.

Albumin concentration about 1 per cent in both experiments.

used to follow the migration of protein zones in electrophoresis or chromatography at constant pH, provided that the indicator used does not combine irreversibly or denature the proteins present. It is usually possible, for a given buffer, to select a suitable indicator which has a considerable "protein error" at this particular pH (Clark, 1928). The indicator is incorporated in the buffer and colour changes indicate the location of the different protein zones in the column.

The long separation time reported in this preliminary study may be a serious drawback. It should, however, be observed that in several of these experiments the separation time would have been reduced by approximately a third if more efficient cooling machinery had been available at the time when

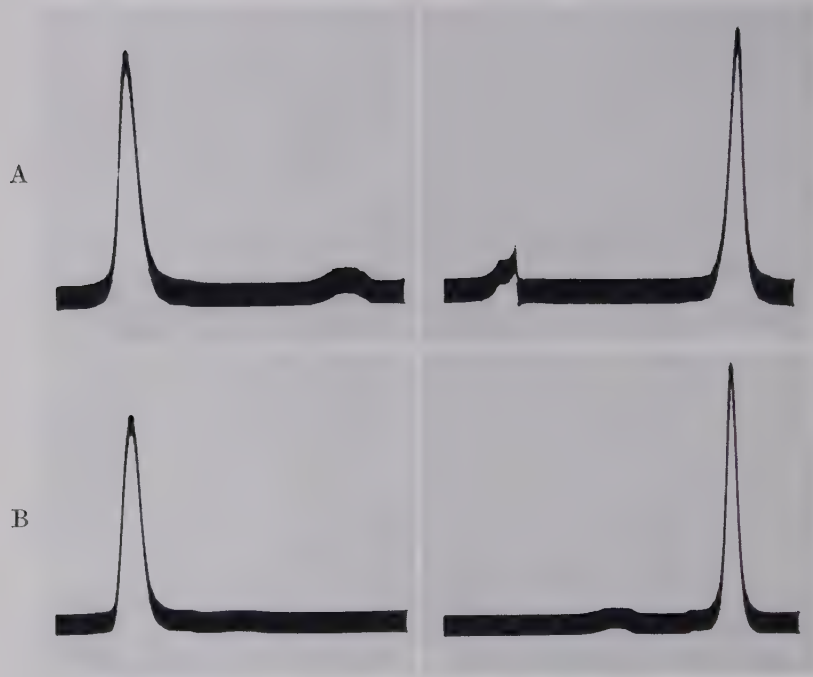

FIG. 51. Moving boundary patterns obtained with the albumin fraction from tube no. 11 in Fig. 46.

A. Pattern obtained in barbiturate buffer at pH 8.6, ionic strength $\mu = 0.1$; 154 min. at 20 mA. Mobility $= 6.8 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}. Ascending side to the right.

B. Pattern obtained in phosphate buffer at pH 7.6, ionic strength $\mu = 0.1$; 165 min. at 25 mA. Mobility 5.0×10^{-5} cm^2 volt^{-1} sec^{-1}. Ascending side to the right.

Albumin concentration about 1 per cent in both experiments.

these preliminary experiments were made. More than 1.5 kilowatts could only be passed through the column for considerable periods of time if enough ice was added to the cold bath which was also cooled by the refrigerator.

The fraction 1 obtained in the experiment reported in Fig. 43 consists of orosomucoid (Winzler, 1955). Synonyms for this protein are M1 (Mehl, Golden, and Winzler, 1949), MP-1 (Weimer, Mehl, and Winzler, 1950) and acid glycoprotein (Schmid, 1953). Nilsson and Yamashina (1958) have observed that orosomucoid exhibits an anti-tromboplastic activity. Boström, Rodén, and Yamashina (1958) found a high turnover rate for the orosomucoid of guinea pigs. Its high content of sialic acid was reported by Werner and Odin (1952). A semi-quantitative estimation of its serum content may be obtained through two-dimensional paper electrophoresis (Markham, Jacobs, and Fletcher, 1956; Giri, 1957).

The present study has shown that the serum fraction obtained in electrophoresis at pH 4.5, and designated in the literature as M2, has different compositions depending on the buffer employed in the fractionation. The fraction 2 obtained in the experiments reported in Figs. 41 and 42 consists of haptoglobin 2–2. In another experiment a patient belonging to the genetic group haptoglobin 1–1 was studied. In this case the haptoglobin did not separate completely from albumin and the sedimentation experiment only showed one peak. The sedimentation constant was $S = 3.7$ in citrate-phosphate buffer at pH 4.5 (p. 54) when a 1.3 per cent solution was studied.

No attempt to explain the splitting of albumin observed in the experiments reported in Figs. 43 and 45 will be made until further experimental data have been obtained. It is however interesting that the two albumins in fractions 2 and 3 obtained from these experiments show the same sedimentation pattern in both cases. Only a few per cent of fast sedimenting material was found. An implication of this is that the beta-component obtained when fraction 3 (Fig. 45) is studied in paper electrophoresis at pH 9.0 has a sedimentation rate not far from that of albumin.

SUMMARY

A vertical zone electrophoresis apparatus, which has been constructed with special regard to efficient cooling, is described. The dimensions of the separation column allow preliminary fractionation of serum samples of the order 200–400 ml. However, samples as small as 20 ml may also be fractionated. The fractionation should be carried out at a temperature between 0° and +4°C. Narrow passages have been avoided in the construction of the apparatus. The importance of a well-defined zone is stressed.

A method for the application of a zone from below is described.

The relation between zone-spreading, protein concentration, and ionic strength is discussed.

Density gradient experiments may be performed in the separation column both with and without further stabilizing media.

A method for the observation of protein zones, which is based on the "protein error" of certain indicator dyes, is described. It may be used for zone electrophoresis columns and protein chromatography at constant pH.

The use of zone electrophoresis to obtain an "adjusted solution" according to Kohlrausch's "regulating function" is discussed; δ- and ε-boundaries in

moving boundary experiments with single components may thus be suppressed.

The importance of starting the separation with a fresh sample is stressed. In order to minimize contamination with microorganisms, EDTA has been added to some of the buffers used.

An ultrafiltration unit is described.

In certain buffers it is possible to isolate serum components such as orosomucoid, haptoglobin, or albumin in a single fractionation. Enough material may be obtained in the first experiment to make possible a second fractionation using chromatography or electrophoresis in another buffer. Starch gel electrophoresis may then be used for characterizing microheterogeneities in isolated fractions.

From serum proteins even minor components of the order of 0.1–0.5 per cent may be isolated from individual sera. These minor components may be further characterized with regard to the amino-acid composition, the hexose, hexosamine, and sialic acid contents, and also such physical constants as S/D and the radius of gyration may be obtained with fractions isolated from individual sera. Some methodological details in connection with determinations of hexosamine and sialic acid are discussed.

By performing the electrophoresis in a citrate-phosphate buffer with EDTA at pH 4.4 for seven days and then gradually adjusting the pH from 4.4 to 6.5 by means of an electrode-reaction during the following day, it is possible to obtain directly from the column 340 ml of a 1 per cent albumin solution from 100 ml of the original serum. This albumin fraction is homogeneous in the ultracentrifuge and also in free electrophoresis at pH's 4.4, 4.5, 7.5, and 8.9. At pH 4.0, however, the fraction shows a certain splitting in moving boundary electrophoresis after one hour.

Preliminary experiments with sera from patients with rheumatoid arthritis are reported. Increased orosomucoid and haptoglobin levels have been found.

The kind of separation obtained at about pH 4.5 is markedly influenced by the buffer ions used. In citrate-phosphate buffer it is thus possible to obtain the haptoglobin migrating in front of the albumin fraction, while in acetate buffer at about the same pH a quite different form of separation is obtained. In the latter case the albumin peak is divided into two and the first contains albumin and slow alpha-2 globulin as shown in sedimentation experiments and in gel electrophoresis.

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